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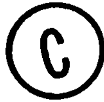
THE UNIVERSITY OF ALBERTA

STUDIES ON THE SUBUNIT STRUCTURE AND THE ACTIVE SITE SEQUENCE OF SUCCINYL COENZYME A SYNTHETASE FROM *E. COLI*

by

TUSN-TIEN WANG



A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "STUDIES ON THE SUBUNIT STRUCTURE AND THE ACTIVE SITE SEQUENCE OF SUCCINYL COENZYME A SYNTHETASE FROM *E. COLI*" submitted by TUSN-TIEN WANG in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The reaction catalyzed by succinyl coenzyme A synthetase of *Escherichia coli* involves the intermediate participation of a phosphorylated form of the enzyme. We have confirmed the nature of the phosphorylated residue as phosphohistidine by a study of the kinetics of acid hydrolysis of the phosphoenzyme, and by the isolation of a phosphohistidine-containing peptide following digestion of the phosphoenzyme with trypsin. The amino acid sequence of this dodecapeptide has been determined to be: met-gly-his(PO_3^{2-})-ala-gly-ala-ile-ile-ala-gly-gly-lys. This is the first example of determination of the amino acid sequence of a phosphohistidine-containing peptide. We describe a simple chromatographic method for the selective purification of peptides containing phosphohistidine, which may also be of utility in studies of proteins containing other phosphorylated amino acid residues.

Previous studies using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate had indicated that the enzyme has two species of subunits, perhaps in an $\alpha_2\beta_2$ structure. Both qualitative and quantitative N-terminal residue determination substantiate the proposed subunit structure. Moreover, the amino acid compositions of the isolated subunits and electrophoretic mapping of peptides derived from the enzyme confirm the $\alpha_2\beta_2$ subunit structure.

We have found that succinyl coenzyme A synthetase is markedly resistent to inactivation by trypsin when ATP is present. Similar protection by ATP is also seen in inactivation studies with three other

proteases. These results are interpreted in terms of a substantial conformational change which accompanies the phosphorylation of the enzyme. Large differences in stability between the phosphorylated and nonphosphorylated enzyme forms are also seen during storage at 4°C. The data suggest that the instability of the dephosphorylated enzyme during such storage may be correlated to proteolytic degradation.

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LIST OF ABBREVIATIONS

ADP	-	adenosine 5'-diphosphate
ATP	-	adenosine 5'-triphosphate
A_{280}	-	optical density at 280 nanometers
CoA	-	Coenzyme A
Dansyl chloride (DNS-Cl)	-	1-Dimethylaminonaphthalene-5-sulfonyl chloride
DNS	-	1-Dimethylaminonaphthalene-5-sulfonyl
DNS-Met(O)	-	Dansyl methionine sulfoxide
DNS-Met$(O)_2$	-	Dansyl methionine sulfone
E. coli	-	Escherichia coli
EDTA	-	Ethylenediaminetetraacetate
E-P	-	phosphorylated succinyl CoA synthetase
GTP	-	guanosine-5'-triphosphate
ITP	-	inosine-5'-triphosphate
mc	-	millicurie
M.W.	-	molecular weight
NDP kinase	-	nucleoside diphosphokinase
P_i	-	inorganic phosphate
PITC	-	phenylisothiocyanate
QAE-Sephadex	-	Diethyl-(2-hydroxypropyl)aminoethyl-Sephadex

LIST OF ABBREVIATIONS (Continued)

SCS	-	succinyl Coenzyme A synthetase
SDS	-	sodium dodecyl sulfate
S_{obs}	-	sedimentation coefficient at 20°C in 0.05 M Tris-Cl buffer at pH 7.2
TFA	-	trifluoroacetic acid
TLC	-	thin layer chromatography
TLCK	-	tosyl-L-lysine chloromethyl ketone
TPCK	-	L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone
Tris	-	tris (hydroxymethyl) aminomethane
μℓ	-	microliter

CHAPTER I

INTRODUCTION

Catalysis via covalent intermediates is one of the most important mechanisms in enzymic reactions. There are many enzymes in which covalent participation of side chains of amino acid residues appear to be involved in the catalytic function. Evidence for the existence of such intermediates has been obtained by a number of approaches, including the observation of exchange reactions and the actual isolation of intermediates. A literature search of the covalent enzyme-substrate intermediates and means for their detection has recently been presented by Bell and Koshland (1).

In the case of phosphorylated enzyme intermediates, there are examples of phosphorylation of the hydroxyl group of serine, of the imidazole group of histidine and of the carboxyl group of aspartic acid or glutamic acid in certain phospho-transferases. Some examples in which the phosphoryl intermediate has been identified with a specific amino acid side chain are shown in Table I. The nature of phosphoenzyme intermediates can be classified into three groups according to stability at extremes of pH:

(1) Protein-bound, alkali-labile phosphate:

The only phosphorylated amino acid residue known in this group is O-phosphoserine. It is very stable to acid at 100°C but is unstable in alkali (2). Enzymes containing phosphoserine as an intermediate are shown in Table I. It is also anticipated, of course, that O-phosphothreonine residues would exhibit the same stability characteristics.

(2) Protein-bound, acid-labile phosphate:

This group contains two phosphoamino acids, phosphohistidine and phospholysine. Two isomers of phosphohistidine, 1-phosphohistidine and 3-phosphohistidine have been obtained from living systems (6,11,17). In 1962, P. D. Boyer and colleagues (18) isolated phosphohistidine from a mitochondrial fraction which was rapidly labelled by $^{32}P_i$ during oxidative phosphorylation. This resulted in the first observation of the presence of a phosphorylated imidazole structure in material isolated from living systems. Now, phosphohistidine has been found to occur in peptide linkages in a number of phosphoproteins, as shown in Table I.

Another acid-labile residue is phospholysine. Walinder (11,17,56) had isolated N-ε-^{32}P-phospholysine, together with phosphohistidine, from yeast, bovine liver and human erythrocytic NDP kinase after incubation with [^{32}P] ATP. Subsequently, 1-phosphohistidine was identified as the main radioactive component. The biological significance of N-ε-phosphoryl-lysine cannot be judged at present. One possible origin of N-ε-^{32}P-phosphoryl-lysine may be the active sites of other enzymes which are intermediately phosphorylated during their action, as has been suggested in the case of phosphohistidine.

(3) Protein-bound, acid- and alkali-labile phosphate:

This group contains acyl phosphates, such as phosphorylated derivatives of glutamic acid and aspartic acid residues. Acetyl phosphate has been synthesized and studied by Koshland (19), where it was found that acetyl phosphate is labile to both acid and base. The pH stability curve of β-aspartyl phosphate (20) is very similar to that of acetyl phosphate (19). Table I gives some enzymes which

TABLE I

AMINO ACID IDENTIFIED WITH COVALENT PHOSPHOENZYME INTERMEDIATES

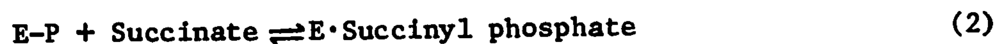
Amino Acid	Enzyme	Reacting Group	Covalent Intermediate
I. Serine	*E. coli* alkaline phosphatase (3,77) phosphoglucomutase (4)	OH	phosphoserine
II. Histidine	Succinyl coenzyme A synthetase (5,6) Phosphoglyceromutase (7) Glucose-6-phosphatase (8) Acid phosphatase (9) Nucleoside diphosphokinase (10,11) ATP-citrate-lyase (12)	imidazole	phosphohistidine
III. Glutamic acid or Aspartic acid	Na^{+} K^{+} - ATPase (13) phosphoglycerate kinase (14) ATP-citrate lyase (15) Acetate kinase (16)	COOH	carboxyl phosphate

have carboxyl phosphate intermediates, all of which show pH-stability properties similar to the above model compounds.

Among enzymes in which phosphohistidine has been identified, SCS from *E. coli* was the first to be subjected to an appropriate pre-steady state kinetic study to establish the obligatory participation of the phosphoenzyme in the catalytic mechanism (21).

SCS (E.C.6.2.1.5) from *E. coli* has a molecular weight of about 140,000 daltons (22). It catalyzes the following reactions (21,23,24).

E + ATP ⇌ E-P + ADP (1)



E-P + Succinate ⇌ E·Succinyl phosphate (2)

E·Succinyl phosphate + CoA ⇌ E + Succinyl CoA + P_i (3)

Sum: ATP + Succinate + CoA ⇌ Succinyl CoA + ADP + P_i

This reaction is fully reversible and requires Mg^{++} ion. This is the reaction of the tricarboxylic acid cycle which involves "substrate-level" phosphorylation to form ATP. SCS from pig heart has a molecular weight of approximately 75,000 daltons (25,26) half the size of the *E. coli* enzyme, and reacts with GTP or ITP instead of ATP.

The isolation of phosphohistidine in SCS was achieved by Kreil and Boyer (6) in 1964 . They labeled this enzyme by incubation with $^{32}P_i$ and succinyl CoA in the presence of Mg^{++} ion. E-^{32}P, together with synthetic phosphohistidine, were incubated in 3 N NaOH for 80 minutes at 100 - 105°C. The sample was dried and then chromatographed. The principal radioactive component co-migrated with added synthetic phosphohistidine and the specific radioactivity was constant throughout the peak. This experiment identified the ^{32}P-labelled component in the *E. coli* succinyl CoA synthetase preparation as bound phosphohistidine. The possibility of

phosphoryl migration during alkaline proteolysis (12,15) remained, however, an open question.

The question then arises as to whether phosphohistidine is really involved as an intermediate species in the enzyme's catalytic function. Bridger _et al_. (21) have established that the phosphohistidine-containing enzyme meets the kinetic criteria required for an obligatory intermediate in the catalysis. The expected kinetic behavior for phosphoryl enzyme intermediates can be explained by the reaction:

The reaction may be rewritten as:

If E-P is an obligatory intermediate, it follows that in the initial phase of the reaction:

(1) E-P must be formed at least as rapidly as the overall steady state reaction rate.

(2) E-P must reach its steady state level no later than when the overall reaction reaches its steady state velocity.

By means of rapid mixing and quenching experiments in both the forward and reverse directions of the SCS reaction, it was shown that the above criteria are met (21), establishing that the phosphoenzyme is a participant on the major catalytic pathway.

In order to confirm that the phosphorylated enzyme contains phosphohistidine, a comparison of the acid hydrolysis of model synthetic phosphohistidine and E-P was made, and the results are shown in Chapter III of this dissertation. Furthermore, we (27) have isolated a

dodecapeptide, containing the active site phosphohistidine, obtained by digestion of the phosphorylated enzyme with TPCK-Trypsin (see Chapter IV of this dissertation). We describe a method for the selective purification of this peptide which may have utility in studies of other phosphoproteins such as those shown in Table I.

By incubation with excess substrates, SCS from E. coli can be phosphorylated by at least one, and perhaps approaching two phosphoryl groups per mole of enzyme (7,3,29,30). This result suggested the possibility of subunit interaction. Enzyme dissociation by p-mercuribenzoate (22) was the earliest confirmation of this possibility. Grinnell et al. (31) had detected subunits by immunodiffusion measurements, but could make no firm conclusions about the number, size, or identity of such subunits. Leitzmann et al. (32) showed the dissociation of the enzyme into four apparently identical subunits by treating with p-mercuribenzoate or SDS, or with urea or guanidine hydrochloride solution and by alkylation with iodoacetamide or succinylation with succinic anhydride in urea solutions. However, Bridger (33) has clearly demonstrated that SCS from E. coli is composed of two species of subunits, by means of polyacrylamide gel electrophoresis in the presence of SDS. His data are easily reconciled with an $\alpha_2\beta_2$ structure. The smaller of the two subunits (α) has a molecular weight of about 29,500 and bears the phosphohistidine residue. The other one (the β subunit) has a molecular weight of approximately 38,500. The proposed $\alpha_2\beta_2$ structure would predict an oligomeric molecular weight of approximately 136,000, comfortably near the value of 141,000 determined by sedimentation equilibrium (22).

This type of structure is also confirmed by N-terminal studies, both qualitative and quantitative, which are reported in this dissertation in Chapter V. The amino acid composition of SCS from *E. coli* is also given.

An intriguing feature of the kinetics of SCS is the phenomenon of "substrate synergism" (21). For example, SCS will catalyze a partial reaction of an ADP $\longleftrightarrow$ ATP exchange which reflects the step in the reaction involving the formation of phosphoenzyme. In the absence of other substrates, the ADP $\longleftrightarrow$ ATP exchange rate is slow, but it is increased markedly by the presence of the other four substrates. This implies that the active site is fully "active" only when all substrate binding sites are filled. The precise explanation for substrate synergism is still not known, but it may possibly relate to substrate-induced conformational changes. The best evidence for such changes is provided by comparison of the reactivity of the phosphorylated and dephosphorylated enzymes. The enzyme is very unstable to storage following dephosphorylation, but is very stable with ATP or P_i present or following phosphorylation. The dramatic effects of these reagents extend to the stability of the enzyme in the presence of proteases, such as trypsin. Some of these properties are also reported in this thesis, and are interpreted in terms of conformational changes which occur upon phosphorylation of the enzyme.

CHAPTER II

GENERAL MATERIALS AND METHODS

I. Materials

A. Chemicals and Solvents

Sephadex G-25 (50-150 μ), G-50, G-100 (40-120 μ) and QAE-Sephadex A-25 (40-120 μ), A-50 (40-120 μ) were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Ion exchange resins AG1-X2 (Dowex 1-X2) (100-200 mesh) and AG50W-X2 (200-400 mesh) were purchased from Bio-Rad Laboratories, Richmond, California, U. S. A. N-ethylmorpholine was obtained from Eastman Organic Chemicals, Rochester, New York, U. S. A. and was redistilled prior to use. Tris (ultrapure) and urea (ultrapure) were purchased from Mann Research Laboratories. Dansyl amino acids were obtained from Calbiochem, Los Angeles, California, U. S. A. or were prepared by the method as described by Gray (35). Disc gel electrophoresis supplies were obtained from Canalco. Polyamide thin layer chromatography sheets were obtained from the Cheng Chin Trading Company by way of Allied Scientific Company. All other chemicals in this study were reagent grade and, except where noted, were used without further purification or treatment.

B. Special Reagent

ATP-γ-^{32}P was prepared in 20 mc batches according to the method of Ramaley *et al*. (22), using ^{32}P-orthophosphate obtained from New England Nuclear.

C. Enzymes

Trypsin-TPCK was purchased from Worthington Biochemical Company, Freehold, New Jersey, U. S. A. Hexokinase was obtained from Sigma Chemical Company. An α-lytic protease preparation from Sorangium sp. (36) was kindly donated by Drs. D. R. Whitaker and L. B. Smillie. Pronase (A grade) was obtained from Calbiochem. TLCK-α-chymotrypsin (34) was kindly provided by Dr. L. B. Smillie.

II. Methods

A. Preparation of SCS from *E. coli*

Succinyl CoA synthetase was prepared from succinate-grown *E. coli* (Crooke's strain) essentially according to the method of Leitzmann *et al*. (32). We deviate from their method by substituting QAE-Sephadex A-50 in place of DEAE-Sephadex for ion exchange chromatography, and in a final purification step, passage through a G-100 Sephadex column, in most preparations. The enzyme used in this study was shown to be chromatographically and electrophoretically homogeneous, and had a specific activity in excess of 30 units per mg. The enzyme was routinely stored in 75% $(NH_4)_2SO_4$ at 4°C and is very stable in this condition (see Chapter VII for further comments on stability).

B. Preparation of Phosphorylated SCS

(i) Phosphoenzyme from ATP-γ-^{32}P

The phosphorylated enzyme was prepared by incubating the enzyme (10 mg) with 0.1 mM ATP-γ-^{32}P (7×10^7 cpm/µmole), 10 mM $MgCl_2$, 0.05 M KCl and 0.05 M Tris-Cl (pH 7.2) for 15 minutes in a small volume. E-^{32}P was assayed by the phenol extraction as described by Ramaley _et al_. (22). The reaction mixture was cooled in an ice bath and then passed through a G-50 Sephadex column equilibrated with the same buffer. The labelled protein peak was collected and was found to contain from 0.7 to 1.0 phosphoryl group per 140,000 daltons of protein in different preparations, in keeping with previous observations (22) on the stoichiometry of this phosphorylation reaction.

(ii) Phosphoenzyme from $^{32}P_i$

The phosphorylated enzyme can be obtained from another direction with pure enzyme (10 mg) reacting with 0.3 mM succinyl-CoA, 1 mM $^{32}P_i$ (4×10^7 cpm/µmole), 10 mM $MgCl_2$, 0.05 M KCl and 0.05 M Tris-Cl (pH 7.2) for 15 minutes in a minimal volume. The phosphoenzyme was obtained the same as before by passing through a G-50 Sephadex column.

C. Amino Acid Analysis

(i) Peptide samples

Quantitative determination of the amino acid composition of peptides was made on the Beckman Spinco Model 120C automatic amino acid analyzer with an expanded range card. Prior to analysis, purified peptides from column chromatography were lyophilized and dissolved in 0.05 M N-ethylmorpholine (pH 8.0) and then passed through a 1 x 45 cm

column of G-15 Sephadex equilibrated with that buffer. The peptide solution was again lyophilized and dissolved in a small volume of N-ethylmorpholine buffer. The sample of peptide (about 0.02 - 0.05 μmole) was hydrolyzed in 0.25 ml of constant boiling HCl (5.7 N) for 20 hours at 110°C in evacuated sealed tubes. The hydrochloric acid was then removed in a vacuum desiccator at room temperature, and the sample was then subjected to amino acid analysis.

(ii) Protein samples

SCS was dialyzed in 0.1 mM phosphate buffer (pH 7.2) for two days at 4°C and then lyophilized. Protein samples were divided into 9 test tubes containing about 0.5 mg each, were hydrolyzed in a sealed tube at 110°C for 24, 48, and 72 hours with 2 ml of constant boiling HCl. The amino acid composition of proteins was determined on the Beckman Spinco Model 120B amino acid analyzer, essentially as described by Moore (37). The cysteine and cystine content of the protein were determined as cysteic acid by the method of Moore (38). Tryptophan was measured spectrophotometrically as described by Edelhoch (39). It was a standard procedure to set up triplicate samples for each hydrolysis time. The different hydrolysis times allowed for correction of hydrolytic losses and incomplete hydrolysis of some peptide bonds. The amino acid residues were calculated based upon a molecular weight of SCS of 140,000 daltons.

D. N-terminal and Sequence Determination of Peptides

The "Dansyl-Edman" technique described by Gray (40,41) was used for the determination of N-terminal groups and sequence of the peptides. About 0.1 to 0.2 μmole of peptide in 0.05 M N-ethylmorpholine was evaporated to dryness and dissolved in 150 μℓ distilled

water. The appropriate amount of solution was removed for dansylation.

(i) Dansylation

Approximately 0.005 to 0.01 μmoles of peptide were removed from the above 150 μℓ sample and added to 20 μℓ of 0.2 M $NaHCO_3$ in a small tube and the solution was evaporated to dryness with a water aspirator. Distilled water (20 μℓ) and 20 μℓ of DNS chloride (2.5 mg per ml in acetone) were added, and incubated at 45°C in a desiccator for 1 hour. After drying, 50 μℓ of 5.7 N HCl was added, the tube was evacuated, sealed and incubated at 110°C for 16 to 20 hours. The sample was then opened and evaporated to dryness.

(ii) Degradation procedure

To the peptide solution, 150 μℓ of 5% PITC in pyridine was added and the mixture was flushed with nitrogen gas and incubated in a desiccator at 45°C for one hour. The sample was then transferred to a desiccator at 60°C which contained NaOH and P_2O_5 and was evaporated to dryness using a vacuum pump. Anhydrous TFA (200 μℓ) was added to the residue, and following flushing with nitrogen gas, the solution was incubated at 45°C in a desiccator for 30 minutes. The TFA was removed in a desiccator at 60°C with P_2O_5 and NaOH for 10 minutes. At this stage the peptide is one residue shorter. To each tube 200 μℓ of distilled water was added and the solution was extracted three times with 1.5 ml of n-butylacetate. The top layer was discarded and the lower layer was taken to dryness and dissolved in 150 μℓ of distilled water for the dansylation and the next degradation step.

(iii) Identification of dansyl amino acids

The dansyl amino acids were identified by thin layer chromatography on polyamide layers (42), using the solvent systems described by Hartley (43).

System 1: 1.5% (v/v) formic acid

System 2: Benzene-acetic acid (9:1, v/v)

System 3: Ethylacetate-methanol-acetic acid (20:1:1, v/v)

The acid hydrolysate was dissolved in 50 per cent pyridine (5 μℓ) or acetic acid-acetone (2:3, v/v) and half of the solution was applied to each side of the thin layer plate. The standard dansyl amino acids are applied on one side. Following development, the dansyl amino acids were located with the aid of an ultraviolet lamp.

E. Polyacrylamide Gel Electrophoresis

For analytical runs in the absence of SDS, the general methods and materials described by Ornstein (44) and in Canalco Disc Electrophoresis instructions (1969) were used. The method described by Weber and Osborn (45) was used for electrophoresis in the presence of SDS, using the normal amount of cross-links in the separating gel.

The enzyme (1-2 mg) in 75% $(NH_4)_2SO_4$ was centrifuged down and dissolved in 0.01 M phosphate buffer, pH 7.0, 1% SDS and 1% 2-mercapto-ethanol for 3 hours at 37°C. The solution was then dialyzed against the electrophoresis buffer (45). Electrophoresis was carried out at a constant current (4 amp - 8 amp) per gel tube with a bromophenol blue marker dye.

F. High Voltage Paper Electrophoresis

High voltage electrophoresis at pH 1.8 was performed in a Gilson High Voltage Electrophorator (Model D) equipped with a large fiberglass tank, as described by Dreyer and Bynum (46). The pH 6.5 electrophoresis is essentially according to the method of Michl (47) and Ryle *et al*. (48). The buffer systems and coolants were similar to those described by Ambler (49) except that the toluene was 8 per cent (v/v) with respect to pyridine for the pH 6.5 buffer system. The buffer system at pH 6.5 is pyridine-acetic acid-water (100:3:900, by vol.) and pH 1.8 is formic acid-acetic acid-water (1:4:45, by vol.). The peptides were purified by paper electrophoresis on Whatman No. 1 MM filter paper with sample applied at the rate of 0.3 mg per cm. Peptides were located with the cadmium-ninhydrin reagent of Heilmann *et al*. (50) and the Pauly reagent for histidine (51). The pure peptide was eluted by distilled water in a closed chamber saturated with water vapor.

CHAPTER III

ACID HYDROLYSIS OF PHOSPHORYLATED SCS

I. Introduction

In 1964, Kreil and Boyer (6) labeled SCS from *E. coli* with $^{32}P_i$ and succinyl CoA in the presence of Mg^{++} ion and identified the ^{32}P component in alkaline digests of this enzyme as phosphohistidine. Later, Hultquist and Boyer (52) prepared synthetic 3-phosphohistidine and determined its rate of hydrolysis at different pH's and temperatures. They also identified the phosphohistidine derived from SCS as the 3-isomer. In spite of these observations, some doubt persisted about the nature of the catalytically active residue, since the possibility remained that alkaline hydrolysis of the phosphorylated enzyme was accompanied by migration of the phosphoryl group to a histidine residue from a primary site of phosphorylation. (Such migration has been proposed to account for difficulties in establishing the nature of the phosphorylated residue in ATP-citrate lyase (15)).

The acid hydrolysis of phosphohistidine followed a first order reaction (52). The first order rate equation is:

$$\log (A) = \frac{-kt}{2.303} + \log (A_\circ)$$

The rate constant k may be obtained from a plot of log (A) versus t, the slope of such a plot is $-k/2.303$. This chapter describes some acid hydrolysis properties of ^{32}P-SCS in order to compare these to those of authentic 3-phosphohistidine and other phosphorylated enzymes such as ATP-citrate lyase.

II. Methods

A. Hydrolysis in 0.05 M Citrate-0.05 M Phosphate Buffer at 46°C

E-^{32}P was prepared as described in Chapter II, Section II. B. Hydrolysis of the phosphoenzyme was carried out at 46°C in 0.05 M citrate - 0.05 M phosphate buffers ranging in pH from 2.1 to 4.0. The rate of hydrolysis was measured by following the decrease of the amount of radioactive ^{32}P-protein by means of the phenol extraction procedure. The reaction mixtures (4 ml of buffer and 0.1 ml of E-^{32}P) were neutralized at timed intervals by the addition of 1 ml of water-saturated phenol, buffered with 0.01 M phosphate at pH 8.5, and 5 ml of cold 0.01 M phosphate buffer saturated with phenol, pH 8.5 was quickly added. The samples were chilled in an ice bath for 2 minutes, thoroughly mixed with a Vortex mixer, and centrifuged. The phenol layer was washed several times with phosphate buffer saturated with phenol, pH 8.5 until no ^{32}P remained in the upper aqueous layer. The phenol layer was dried in a planchet for counting.

B. Hydrolysis in HCl Solution

The hydrolysis of E-^{32}P was carried out in HCl solutions ranging in concentration from 0.1 N to 2.5 N at 48°C. The rate of hydrolysis of E-^{32}P at concentrations of HCL exceeding 3 N was very fast and therefore not suitable for study by conventional methods. The procedure is the same as given in above IIA except only 3 ml of HCl solution was used.

III. Results

A. Rates of Hydrolysis of Phosphorylated SCS

(i) Hydrolysis in 0.05 M citrate - 0.05 M phosphate buffer at 46°C

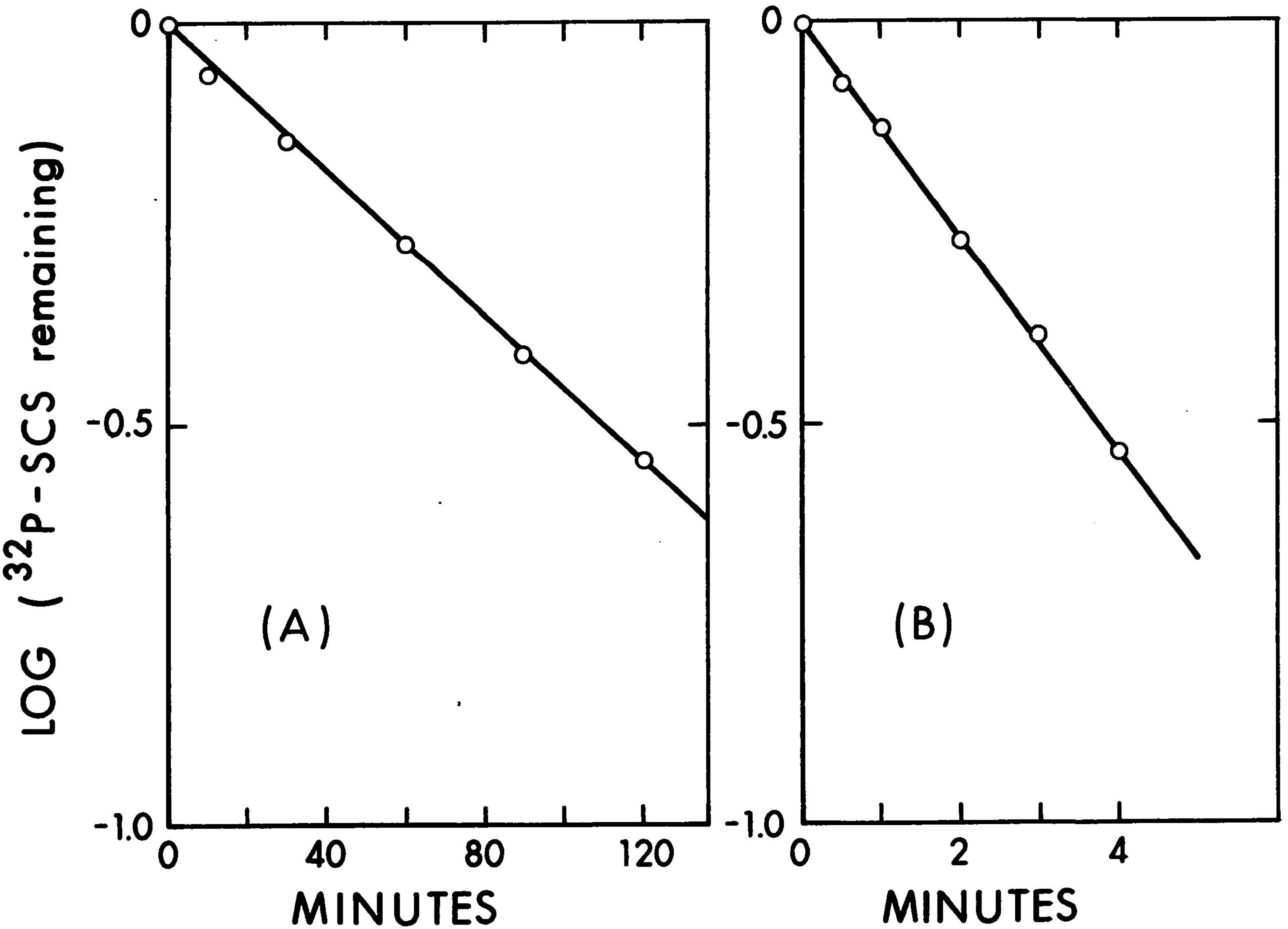
The data shown in Figure 1 (A) is for a hydrolysis at 46°C in 0.05 M citrate - 0.05 M phosphate buffer, pH 3.0. From the slope, we got the first order rate constant at this pH. Identical figures can be obtained at different hydrogen ion concentrations. At all hydrogen ion concentrations studied, the plots of logarithm of residual ^{32}P-protein versus time were linear. The straight lines are calculated by the use of a least squares program written for the Olivetti-Underwood Programma 101. The first order rate constants in 0.05 M - citrate - 0.05 M phosphate buffer are shown in Table II.

(ii) Hydrolysis in HCl solution at 48°C

An example of data obtained for a hydrolysis at 48°C in 0.5 N HCl is shown in Figure 1 (B). The straight line is fitted by computer program as before. The first order rate constants at different concentrations of HCl are shown in Table III.

In the preceding cases, phosphorylated protein was prepared from enzyme reacting with ATP-γ-^{32}P. In order to see whether there is an unique phosphorylated enzyme, we prepared the phosphorylated protein by reaction of SCS with succinyl CoA and $^{32}P_i$ in the presence of Mg^{++} ion. The hydrolysis is carried out the same way as before. All of the data were similar to those of Table II and Table III, within the limits of experimental error. Therefore SCS appears to be phosphorylated in either direction at the same residue.

FIGURE 1: Hydrolysis of ^{32}P-SCS. (A) 0.05 M citrate - 0.05 M phosphate buffer, pH 3.0 at 46°C (B) 0.5 N HCl at 48°C. The phosphorylated protein was measured by the phenol extraction method of Ramaley *et al*. (22).



LOG (^{32}P - SCS remaining)
0
-0.5
-1.0
(A)
0
40
80
120
MINUTES
(B)
0
2
4
MINUTES

TABLE II

ACID HYDROLYSIS OF ^{32}P-SCS IN CITRATE-PHOSPHATE BUFFER AT 46°C

Conditions	k (min^{-1}) for ^{32}P-SCS	k (min^{-1}) for ATP-citrate lyase-^{32}P *
pH 2.1	0.0266	
pH 2.3	0.0212	
pH 2.5	0.0166	0.018
pH 2.7	0.0133	
pH 3.0	0.0104	
pH 3.4		0.0097
pH 4.0	0.0051	

* From G. L. Cottam and P. A. Srere (12).

TABLE III

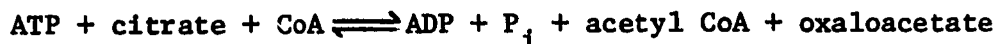
ACID HYDROLYSIS OF ^{32}P-SCS IN HCl SOLUTION AT 48°C

Conditions	k (min^{-1}) of ^{32}P-SCS
0.1 N	0.055
0.5 N	0.30
1.0 N	0.59
1.5 N	1.06
2.0 N	1.65
2.5 N	2.18

IV. Discussion

SCS has a phosphohistidine residue involved in its catalytic function (6,21). The first order rate constants for hydrolysis of phosphorylated protein in Table II and Table III are lower than the rate of hydrolysis of 3-phosphohistidine (52). Plots of k against hydrogen ion concentration for both phosphorylated SCS and 3-phosphohistidine (52) are very similar, as shown in Figure 2. This confirms that SCS has a phosphohistidine at its active site and rules out the artifact of migration from a dissimilar residue to histidine during alkaline proteolysis. The reason for slightly lower k values for SCS may be due to the environment around the region of the active site.

ATP-citrate lyase of rat liver catalyzes the reaction of:



$$\text{ATP} + \text{citrate} + \text{CoA} \rightleftharpoons \text{ADP} + \text{P}_i + \text{acetyl CoA} + \text{oxaloacetate}$$

It has many similarities to SCS, including a phosphoenzyme intermediate. Cottam and Srere (12) phosphorylated ATP-citrate lyase with ATP-γ-^{32}P and then carried out the alkaline proteolysis. They have identified phosphohistidine in the digest and determined the rate of hydrolysis of phosphorylated ATP-citrate lyase, also obtaining results similar to those expected for phosphohistidine. A comparison of the rate constant for hydrolysis of phosphorylated SCS and phosphorylated ATP-citrate lyase (see Table II), shows that these are almost the same. Mardh _et al_. (53) also identified a rat-liver cell sap protein yielding 3-[^{32}P]-phosphohistidine after incubation with ATP-γ-^{32}P followed by alkaline hydrolysis as ATP-citrate lyase. However, Suzuki _et al_. (15,54) indicated a different site of phosphorylation and identified the phosphorylated residue as γ-phosphoryl glutamate. They found the pH-

stability curve of the phosphorylated ATP-citrate lyase to be very similar to that of aspartyl phosphate (20) which is acid- and alkaline-labile. The reason for the discrepancy between these results and those of Cottam and Srere is not understood. One possibility is that all phosphoamino acids may be derived from the active site. Bridger (55) suggested that ATP-citrate lyase may have two sites capable of undergoing phosphorylation. One of them may be expected as catalytic intermediate and another may be due to "accidental" phosphorylation.

FIGURE 2: Effect of hydrogen ion concentration on rate constant k for ^{32}P-SCS and 3-phosphohistidine hydrolysis.

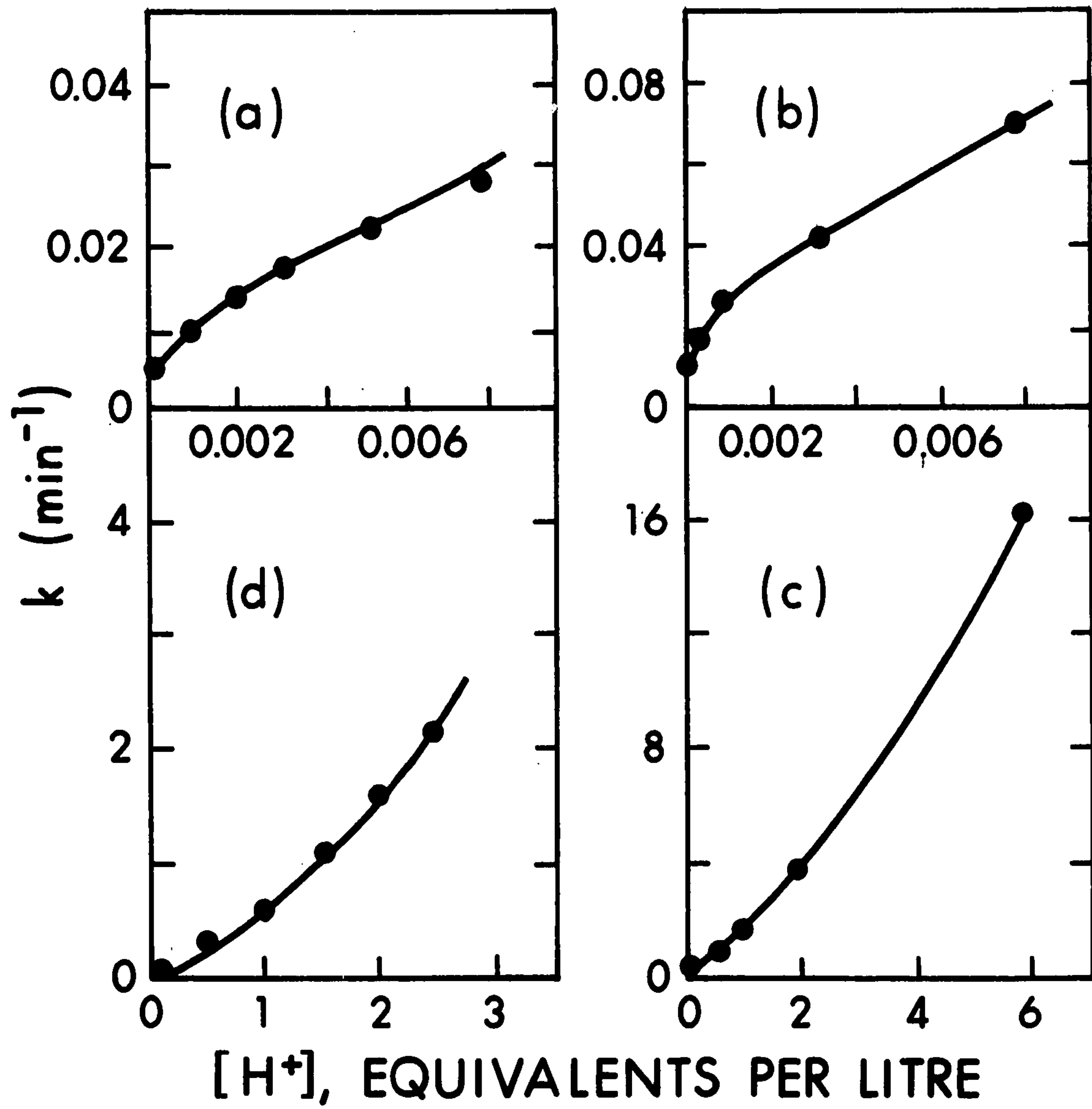
(a) 0.05 M citrate - 0.05 M phosphate buffer at 46°C (^{32}P-SCS).

*(b) 0.05 M citrate - 0.05 M phosphate buffer at 46°C (3-phosphohistidine).

*(c) HCl solution at 48.5°C (3-phosphohistidine).

(d) HCl solution at 48°C (^{32}P-SCS).

* Hultquist *et al*. (52).



(a)
(b)
(c)
(d)
0.04
0.02
0
0.08
0.04
0
0.002
0.006
0.002
0,006
k (min⁻¹)
4
2
0
16
8
0
0
1
2
3
0
2
4
6
[H⁺], EQUIVALENTS PER LITRE

CHAPTER IV

ACTIVE SITE SEQUENCE CONTAINING THE PHOSPHOHISTIDINE RESIDUE OF E. COLI SCS

I. Introduction

SCS of *E. coli* is known to involve a 3-phosphohistidine residue in its catalytic mechanism. The isolation, kinetic studies and acid hydrolysis characteristics of ^{32}P-SCS in Chapter III all confirm the existence of phosphohistidine in the enzymic reaction. The properties of ^{32}P-phosphorylated SCS in acid condition give us a valuable method for isolation of phosphohistidine containing peptides. By making use of the acid lability, we have devised a method using ion exchange column chromatography to isolate a dodecapeptide (27), containing the active site phosphohistidine obtained by digestion of the phosphoenzyme with TPCK-trypsin. Its amino acid sequence has been determined to be: Met-gly-his(PO_3^{2-})-ala-gly-ala-ile-ile-ala-gly-gly-lys. This method may have utility in studies of other phosphoproteins.

II. Methods

A. Digestion of Phosphoenzyme by TPCK-Trypsin

In a typical protocol, 120 mg of SCS was incubated with ATP-γ-^{32}P (5×10^8 cpm/μmole) according to the method given in Chapter II, with 0.05 M Tris-Cl and 0.05 M KCl, pH 8.1 buffer in the Sephadex column. To the phosphoenzyme (15 ml) was then added 1.2 mg of TPCK-trypsin (1% by weight) dissolved in 0.6 ml of the same buffer

(0.05 M Tris-Cl - 0.05 M KCl, pH 8.1). Digestion was carried out at 37°C for 5 hours, after which the reaction mixture was placed on a QAE-A-25 anion exchanger column as outlined in Results.

B. Desalting of Phosphohistidine-containing Peptides

Because of the acid lability of the N-P bond of the phosphohistidine residue, we have found it unsatisfactory to carry out chromatography of the peptides in volatile buffers followed by lyophilization to accomplish desalting -- substantial hydrolysis of the phosphohistidine residue takes place during such lyophilization. A suitable alternative procedure which we have adopted throughout this study involves the passage of the pooled solution of peptide, obtained from ion exchange chromatography, through a 2.5 x 100 cm column of G-25 Sephadex equilibrated with 0.05 N ammonium hydroxide. Subsequent lyophilization of the peptide may then be accomplished with little or no attendant hydrolysis.

III. Results

A. Isolation of [^{32}P]-Phosphopeptide

(i) QAE-A-25 chromatography

The mixture of peptides obtained by proteolysis of 120 mg of ^{32}P-phosphoenzyme was chromatographed on a column of QAE-A-25 Sephadex (1.5 x 112 cm) and the results are given on Figure 3. There are two peaks of radioactivity; the smaller peak (tube 168 - 174) was identified as [^{32}P]-orthophosphate by analysis on a G-25 Sephadex chromatography column (2.5 x 100 cm) and by high voltage electrophoresis. The larger

peak containing the desired peptide was pooled and desalted according to the procedure described under section IIB of this Chapter. This mixture was then subjected to high voltage electrophoresis at pH 6.5 on Whatman No. I MM paper for 60 min at 3KV. The results (Fig. 4) show two histidine-containing peptides, H-1 and H-3 in the direction of the negative electrode which are detectable with Pauly reagent. All of the ^{32}P-radioactivity is lost during the electrophoresis run. The peptides H-1 and H-3 were eluted by distilled water and their amino acid compositions are shown in Table IV. The acid lability of the phosphohistidine residue precludes the possibility of using high voltage electrophoresis at pH 6.5 or less for resolution of the mixture of peptides obtained by tryptic digestion of phosphorylated succinyl CoA synthetase. We have, however, devised an empirical method using ion exchange chromatography at alkaline pH for the purification of the [^{32}P]-phosphopeptide.

(ii) Dowex-1-2X chromatography

Another mixture of peptides was obtained by proteolysis of [^{32}P]-phosphoenzyme and was subjected to chromatography on QAE-A-25 Sephadex. The results were similar to those of Figure 3. The larger peak of radioactivity after desalting and lyophilization was then applied to a column of the anion exchanger AG-1-X2 (1.5 x 112 cm), and the resulting chromatographic pattern is shown on Figure 5. As before, the smaller peak of radioactivity (tube 108 - 115) was identified as ^{32}P-orthophosphate. The larger peak (PH-1, tube 45 - 55) was pooled and desalted, and a small sample was subjected to high voltage electrophoresis at pH 6.5, 3KV for 45 minutes. Only one

FIGURE 3: Separation of peptides obtained by digestion of 120 mg of [^{32}P]-succinyl CoA synthetase with trypsin. Chromatography was carried out on a 1.5 x 112 cm column of QAE-Sephadex A-25, equilibrated with 0.05 M tris-Cl, 0.05 M KCl, pH 8.1. The gradient of increasing KCl concentration is indicated. Tubes 117-127 were pooled and subjected to further analysis.

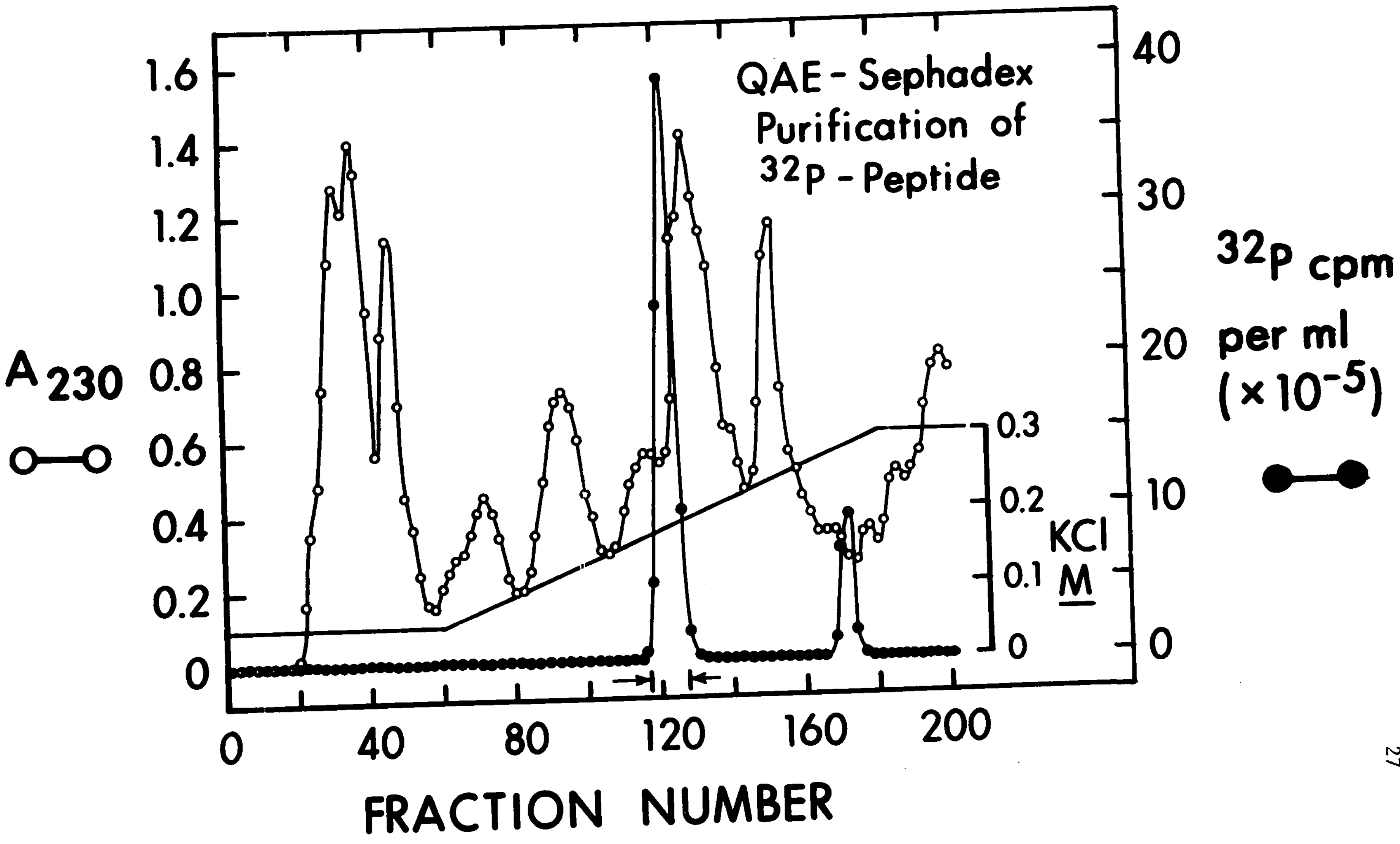
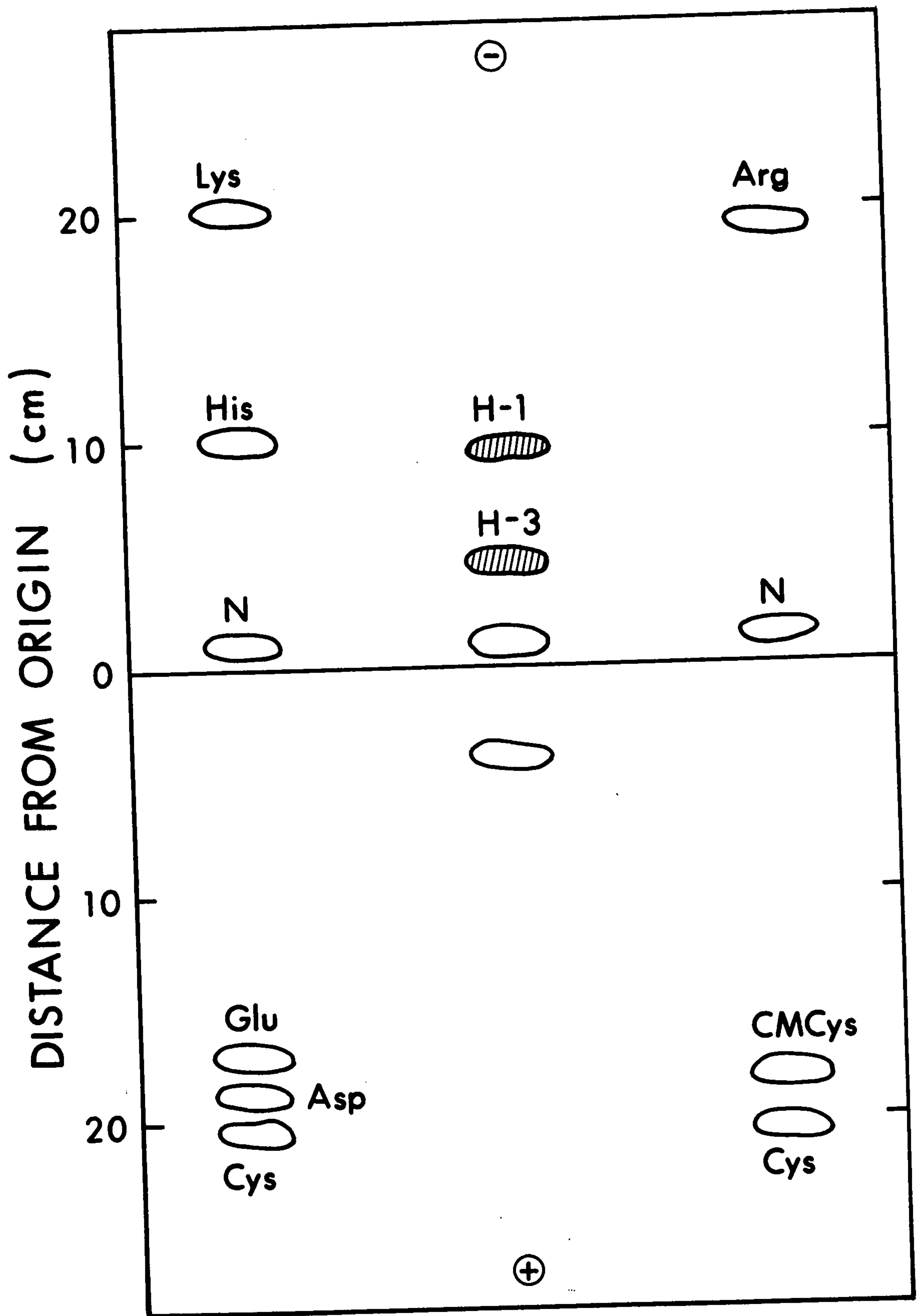
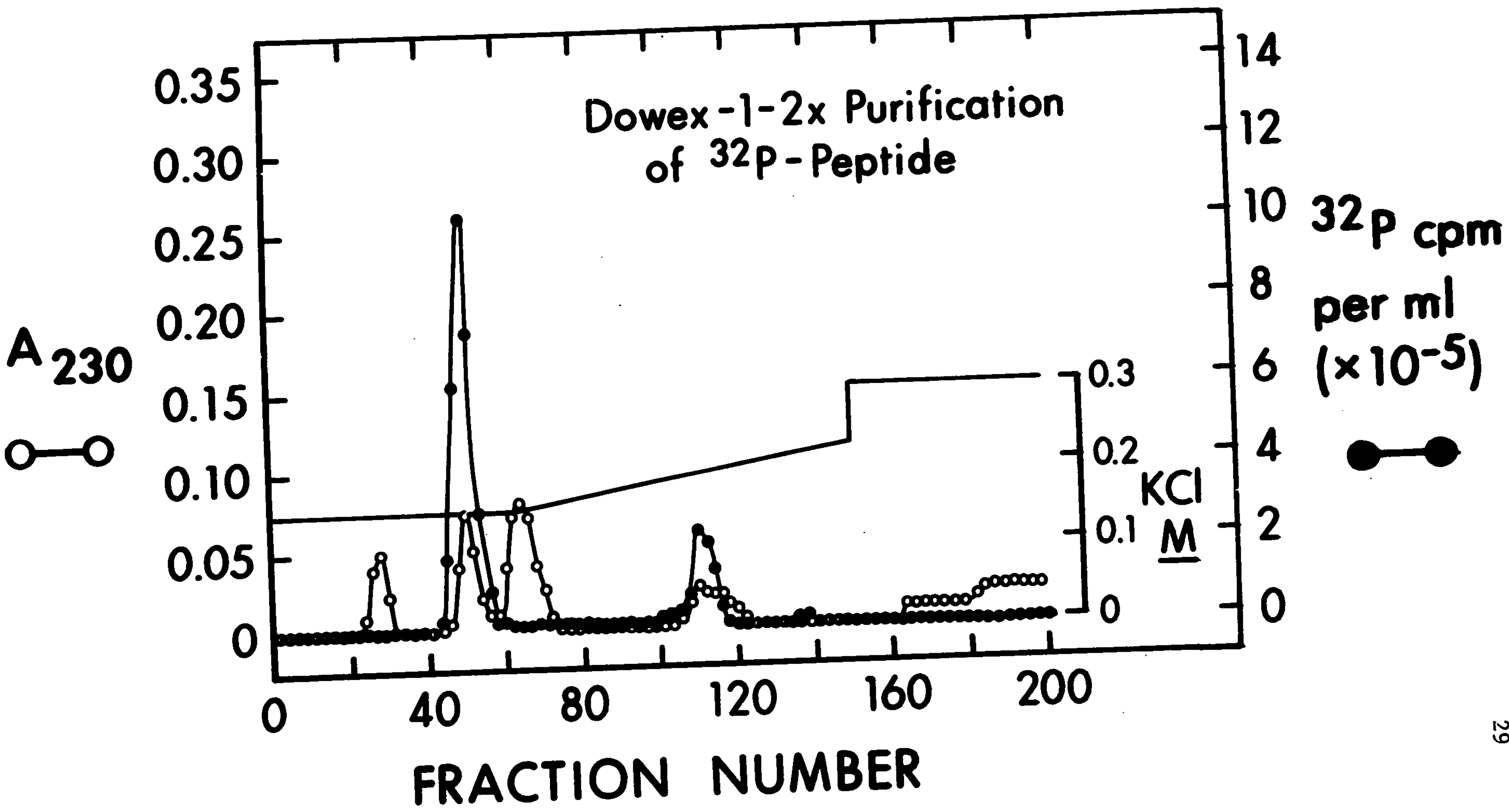

QAE - Sephadex
Purification of
32p - Peptide
A 230
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
40
30
20
10
0
32p cpm
per ml
(× 10-5)
0.3
0.2
0.1
0
KCl
M
0
40
80
120
160
200
FRACTION NUMBER

FIGURE 4: Electrophoretic mobilities of peptides at pH 6.5 (3 KV, 1 hr). Shadowed areas indicate histidine-containing peptides located with Pauly reagent. The standard amino acids are indicated on either side of the sample. N is neutral amino acid and CMCys is s-carboxymethyl-cysteine.



⊖
Lys
Arg
20
His
H-1
10
H-3
N
N
0
DISTANCE FROM ORIGIN (cm)
10
Glu
CMCys
Asp
20
Cys
Cys
⊕

FIGURE 5: Chromatography of mixture of peptides obtained from experiment of Figure 3. A 1.5 x 112 cm column of Dowex-1-2X was equilibrated with 0.05 M tris-Cl, 0.15 M KCl, pH 8.1. Elution was accomplished with a gradient of increasing KCl concentration as shown.



Dowex-1-2x Purification
of 32P-Peptide
A230
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
14
12
10
8
6
4
2
0
32P cpm
per ml
(×10-5)
0.3
0.2
0.1
0
KCl
M
0
40
80
120
160
200
FRACTION NUMBER

peptide was observed whose position was similar to that of peptide H-1. The amino acid composition was determined and N-terminal amino acid analysis by Dansyl chloride method showed only DNS-methionine. Sequence determination as described in Chapter II was therefore undertaken and the results are reported in Table IV (PH-1). The low analysis for isoleucine noted in Table IV is undoubtedly the result of the unusual stability of the ile-ile bond. The peaks of A_{230} at tube 24 - 30 and tube 59 - 71 were subjected to high voltage electrophoresis at pH 6.5. The first peak (tube 24 - 30) shows only one band, which appears to be identical to peptide H-1 as judged by its position on paper and amino acid composition. N-terminal analysis and sequence studies were then undertaken and are shown in Table IV (peptide H-1). The only difference between the peak at tube 24 - 30 and the peak at tube 45 - 55 is one phosphoryl group. Therefore, we devised a "diagonal" method to selectively purify this phosphohistidine-containing peptide which is described in section B of this Chapter. The peak at tube 59 - 71 is not pure, containing the H-3 peptide and some other neutral peptides. The H-3 peptide was isolated on paper by pH 6.5 and pH 1.8 high voltage electrophoresis. The amino acid composition and sequence analysis of this peptide are also reported in Table IV (H-3 peptide). It should be pointed out that neither of the histidine residues of peptide H-3 were derived from phosphohistidine.

(iii) <u>α-lytic protease treatment of PH-1 peptide</u>

Because the identification of α-dansyl-histidine and ε-N-dansyl-lysine is difficult in TLC plates, we sought confirmation of the sequence in the vicinity of the active-site phosphohistidine residues.

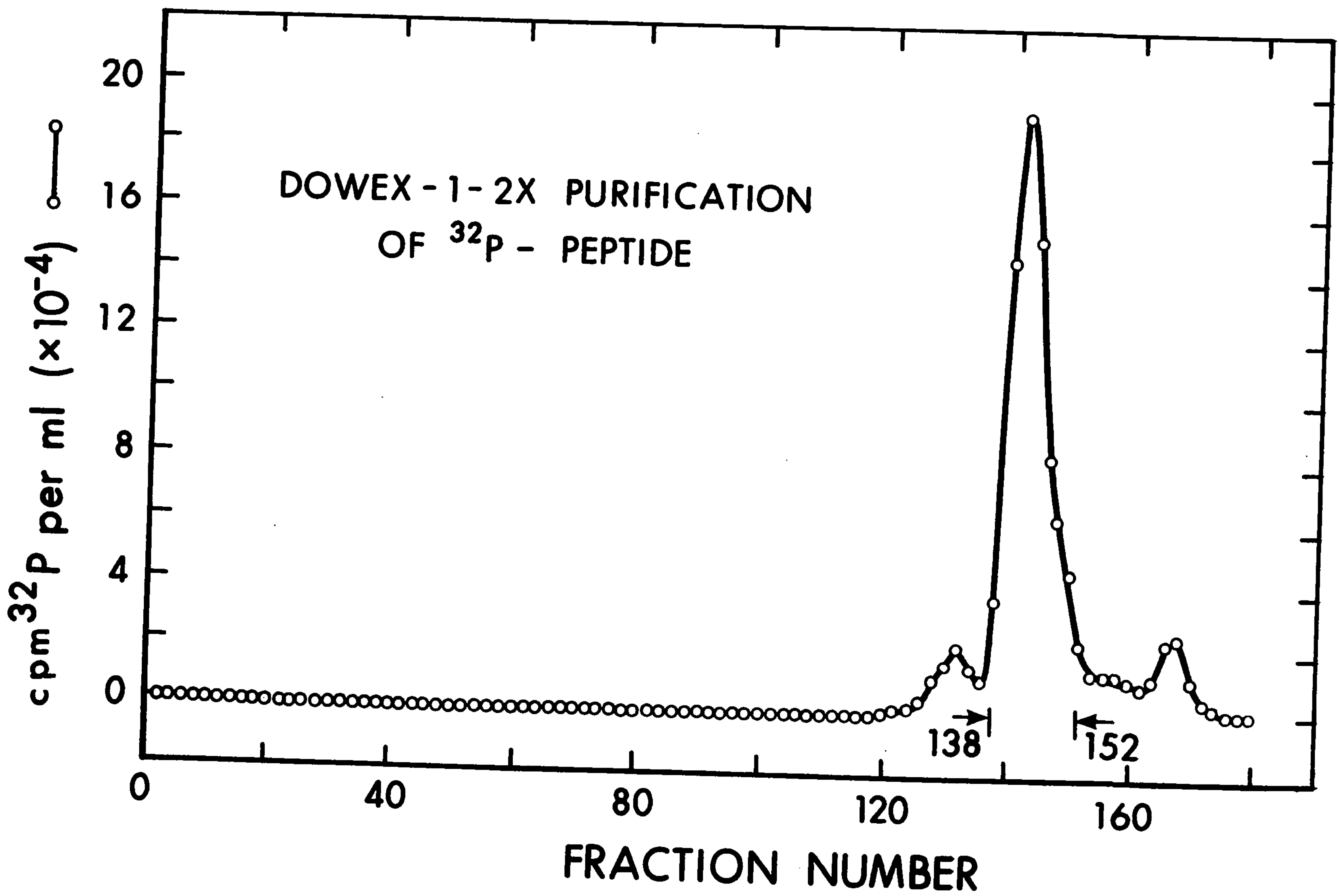
We attempted further proteolysis of peptide PH-1 in the hope of obtaining a shorter peptide containing histidine but having no lysine residue. This was achieved by digestion of a purified ^{32}P-phosphopeptide PH-1 with α-lytic protease (36). The ^{32}P-PH-1 peptide was digested with α-lytic protease (molar ratio 200:1) for 2 hours in a 25°C water bath, followed by chromatography on a 1 x 40 cm Dowex-1-2X (AG-1-X2) column equilibrated with 0.05 M ammonium acetate, pH 8.1. Following elution by a linear gradient of ammonium acetate to a final concentration of 0.5 M, a single big peak of radioactivity (tube 138 - 152, peptide PH-2) was detected as shown in Figure 6. The peak tubes (peptide PH-2) were pooled, desalted and lyophilized. The amino acid composition and sequence of peptide PH-2 are also given in Table IV.

These results confirm the sequence in the vicinity of the phosphohistidine residue found from peptide PH-1, and indicate that α-lytic protease rather specifically catalyzed the hydrolysis of the ala-gly bond resulting in the release of the C-terminal tripeptide from peptide PH-1.

B. "Diagonal" Method of Isolation of ^{32}P-phosphohistidine Peptide

To further confirm the amino acid composition and sequence of the active site peptide, we have developed an alternative procedure for the selective purification of peptides containing phosphohistidine residues. The method takes advantage of the acid lability of such residues, and is related in principle to diagonal electrophoretic methods developed by Hartley and co-workers (43) and to a method devised for isolation of pyridoxal-5'-phosphate-containing peptides (57). The mixture of peptides obtained by tryptic hydrolysis of [^{32}P]-

FIGURE 6: Separation of peptides obtained by digestion of 0.2 μmole of ^{32}P-PH-1 peptide with α-lytic protease. Chromatography was carried on a 1 x 40 cm column of Dowex-1-2X, equilibrated with 0.05 M ammonium acetate, pH 8.1. The gradient starts from tube 20 with 140 ml each of 0.05 M and 0.5 M ammonium acetate. Samples are collected with 1.6 ml per tube.


DOWEX - 1 - 2X PURIFICATION
OF ^{32}P - PEPTIDE
cpm ^{32}P per ml (x10^{-4})
20
16
12
8
4
0
0
40
80
120
160
138
152
FRACTION NUMBER

phosphorylated succinyl CoA synthetase was first subjected to ion exchange chromatography on QAE-Sephadex (Fig. 7, panel A) under conditions the same as those used in the experiment represented by Fig. 3. The peak containing the [^{32}P]-phosphopeptide was pooled, desalted, lyophilized, and then reapplied to an identical QAE-Sephadex column. Elution conditions were the same as before, and the resulting chromatograph is shown on Fig. 7, panel B. The fractions containing the [^{32}P]-phosphopeptide were pooled again, desalted and lyophilized, but this time the powder was taken up in 5 ml of 0.1 N HCl and incubated at 25° for 12 hours to accomplish hydrolysis of the N-P bond of the phosphohistidine residue. The solution was then diluted with 50 ml of water, lyophilized, and reapplied to a third identical QAE-Sephadex column. Following elution as before (Fig. 7, panel C), a new peak at tubes 31 - 35 appeared which was presumed to be the desired peptide now lacking the negatively charged phosphoryl group, hence being bound less strongly to the anion exchanger. The peak (peptide H-1) was pooled and desalted as before. A sample was subjected to high voltage electrophoresis (pH 1.8 and pH 6.5, 3 kV, 45 minutes); only one ninhydrin-positive spot was apparent. The composition and sequence of peptide H-1 were determined and found to be identical to those reported for peptide PH-1 (Table IV).

FIGURE 7: Selective purification of peptide containing potential phosphohistidine residue. In all cases, chromatography was carried out on columns (1.5 x 112 cm) of QAE-Sephadex A-25, previously equilibrated with 0.05 M tris-Cl, 0.05 M KCl, pH 8.1. Elution was accomplished by a linear gradient of KCl from 0.05 M to 0.30 M. Panel A: Separation of peptides obtained by tryptic digestion of 120 mg of [^{32}P]-succinyl CoA synthetase. The indicated fractions were pooled, desalted (by passage through G-25 Sephadex equilibrated with 0.05 M NH_4OH and lyophilization), and were applied to a second column. The chromatogram is shown in panel B. Pooled fractions were then desalted as before, subjected to mild acid treatment (see text), and rechromatographed a third time (Panel C). Fractions containing the desired peptide were pooled as indicated. See text for further details.

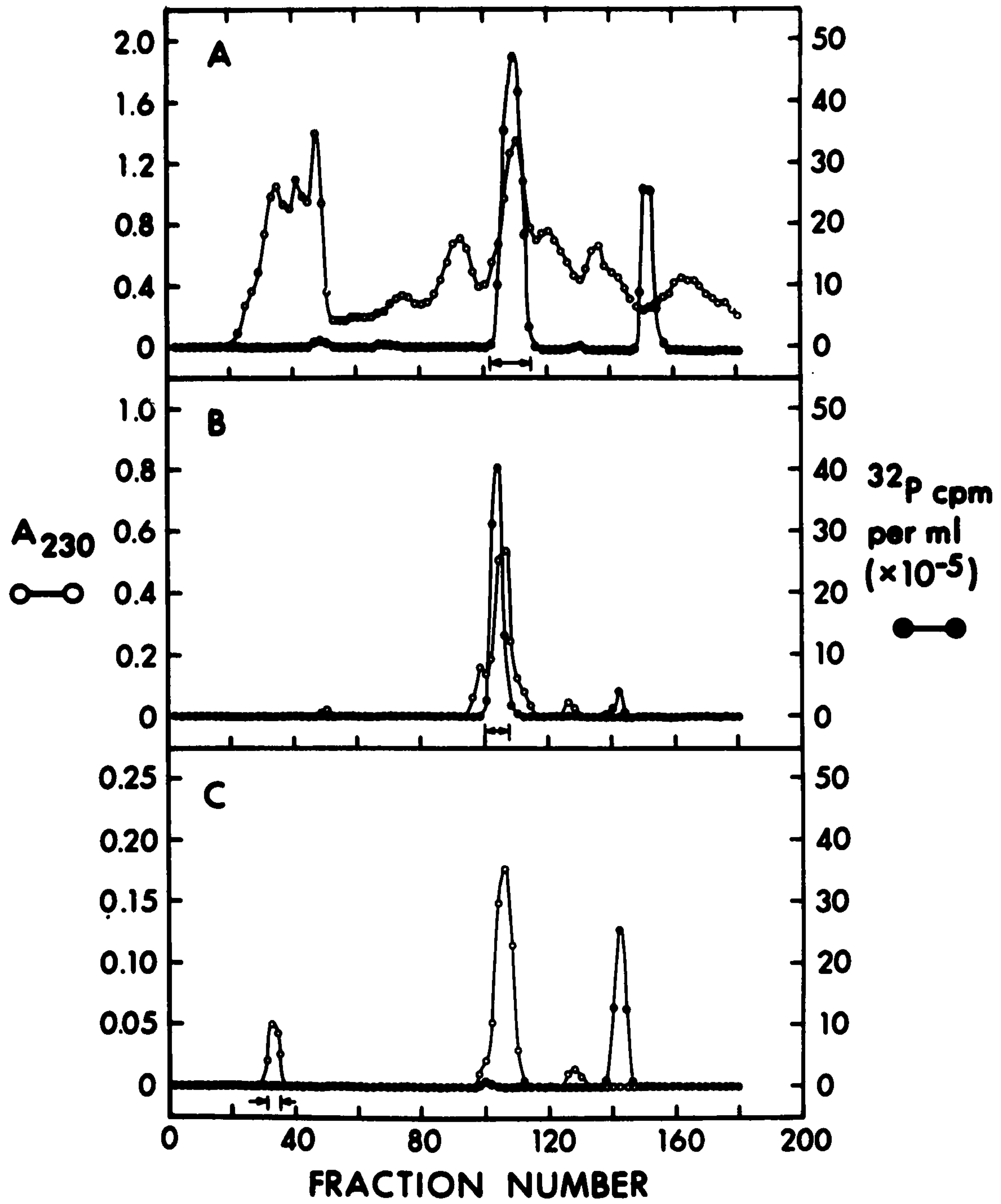

A
B
C
A230
32P cpm per ml (×10-5)
FRACTION NUMBER
0
40
80
120
160
200
2.0
1.6
1.2
0.8
0.4
1.0
0.6
0.2
0.25
0.20
0.15
0.10
0.05
50
40
30
20
10

TABLE IV

AMINO ACID COMPOSITION AND SEQUENCE OF HISTIDINE-CONTAINING PEPTIDES

Peptide												
PH-1	Met -	Gly -	His -	Ala -	Gly -	Ala -	Ile -	Ile -	Ala -	Gly -	Gly -	Lys
	1.02	1.04	1.00	1.03	1.04	1.03	0.60	0.60	1.03	1.04	1.04	1.10
	→	→	→	→	→	→	→	→	→	→	→	→
PH-2	Met -	Gly -	His -	Ala -	Gly -	Ala -	Ile -	Ile -	Ala			
	0.75	1.05	1.00	1.00	1.05	1.00	0.69	0.69	1.00			
	→	→	→	→	→	→	→	→	→			
H-1	Met -	Gly -	His -	Ala -	Gly -	Ala -	Ile -	Ile -	Ala -	Gly -	Gly -	Lys
	1.00	1.01	1.00	1.04	1.01	1.04	0.60	0.60	1.04	1.01	1.01	1.02
	→	→	→	→	→	→	→	→	→	→	→	→
H-3	Val -	Ala -	Glx -	Glx -	Thr -	His -	His -	Leu -	Ile -	Lys		
	1.04	1.00	0.92	0.92	0.90	0.95	0.95	0.88	0.84	1.00		
	→	→	→	→	→	→	→	→	→	→		

IV. Discussion

The sequence which we have determined for peptide PH-1 represents the first known active site sequence for a phosphohistidine-containing enzyme. The sequence contains no features that are readily interpreted in terms of further understanding of the catalytic mechanism. Nevertheless, it will be of great interest to determine whether sequence homology exists between succinyl CoA synthetase and any of the other enzymes noted in Table I which are also thought to involve intermediate participation of phosphohistidine in their catalysis. The possibility of such homology would appear particularly strong in the case of ATP-citrate lyase, which shows close parallels to succinyl CoA synthetase in the nature of both the overall reaction and the proposed citryl phosphate and citryl CoA intermediates (58,59,60).

We believe that our procedure for selective purification of the phosphohistidine-containing peptide may be widely applicable to other phosphorylated proteins. To achieve removal of the phosphoryl residue and the concomitant decrease in net negative charge in peptides containing phosphorylated histidine, lysine, or carboxyl groups, the mild acid treatment we have used should generally suffice. In peptides containing phosphate esters such as phosphoserine, phosphate removal could be accomplished by treatment with alkaline phosphatase. In fact, this approach has been used successfully in the selective purification of a peptide containing the reduced pyridoxal phosphate derivative of *E. coli* glutamate decarboxylase (57).

CHAPTER V

N-TERMINAL GROUP OF SCS

I. Introduction

Many proteins have been shown to consist of several polypeptide chains or subunits. It has been found that SCS of *E. coli* may be dissociated into subunits in the presence of denaturing reagents (31-33). Leitzmann *et al*. (32), on the basis of sedimentation and gel permeation experiments with the alkylated enzyme, suggested that SCS has four subunits of approximately equal size. However, two different species of subunits of SCS have been found by Bridger (33) in SDS-polyacrylamide gel electrophoresis studies. The α subunit, which bears the phosphohistidine residue, has a M.W. of about 29,500 daltons, and the other subunit species (the β subunit) has a M.W. of approximately 38,500 daltons. The number and identity of polypeptide chains can be further studied by N-terminal groups determination, amino acid composition, peptide mapping and sequence studies. In this Chapter, qualitative and quantitative analyses of N-terminal groups are described. The results provide strong confirmation for the suggestion that SCS has an $\alpha_2\beta_2$ type of structure.

II. Methods

A. N-terminal Determination of SCS

(i) Qualitative determination

The qualitative determination of N-terminal group was performed essentially according to the method described by Gray (41),

using the Dansyl chloride procedure. About 10 mg to 20 mg of phosphorylated or non-phosphorylated enzyme, in 5 ml of 8 M urea buffered with 0.5 M $NaHCO_3$, was reacted with 5 ml dansyl chloride solution (20 mg per ml in acetone) and incubated at room temperature overnight. The dansylated protein was precipitated, centrifuged and washed free of salt with distilled water. Constant boiling HCl (5 ml) was added and hydrolysis was allowed to proceed at 110°C for 18 hours. The dansylated amino acid was identified by means of thin layer chromatography as described in Chapter II, section IID (iii). Since identification on polyamide sheets was ambiguous, electrophoresis at pH 4.38 on the flat plate, and pH 1.8 was performed using the methods of Gray (41).

(ii) <u>Quantitative determination</u>

The N-terminal groups of the protein were quantitatively determined by the cyanate method of Stark and Smyth (61,62). Two mls each of N-ethylmorpholine and water were mixed and adjusted to pH 8.0 with acetic acid, made up to 8 M urea and the final volume adjusted to 5 ml. Pure enzyme (about 20 mg to 25 mg) in 2.5 ml of 8 M urea was added to 2.5 ml of the above solution. The enzyme solution was incubated with 300 mg KCNO and the carbamylation reaction was allowed to proceed overnight at 50°C. The reaction mixture was dialyzed against distilled water for 24 hours with several changes of water and then lyophilized.

The cyclization to hydantoins was performed in a solution containing 2 ml of 50% acetic acid and 2 ml of concentrated HCl in an evacuated and sealed test tube at 100°C for one hour. The tube was cooled and opened, and the sample was divided into two parts with 0.1 ml

for amino acid composition analysis and the other 3.8 ml for hydantoins analysis.

The separation of hydantoins from the mixture was achieved using Dowex-50-X2 from which fractions A, B, C-I and C-2 were obtained. The fraction A contains all neutral and acidic amino acid hydantoins, fraction B contains hydantoins of homocitrulline and tryptophan, C-1 fraction contains hydantoin of histidine and C-2 those of arginine. The fractions A, B, C-1 and C-2 were subjected to hydrolysis, and the hydrolysate was quantitatively analyzed with the amino acid analyzer.

B. Determination of N-terminal Residues of Subunits

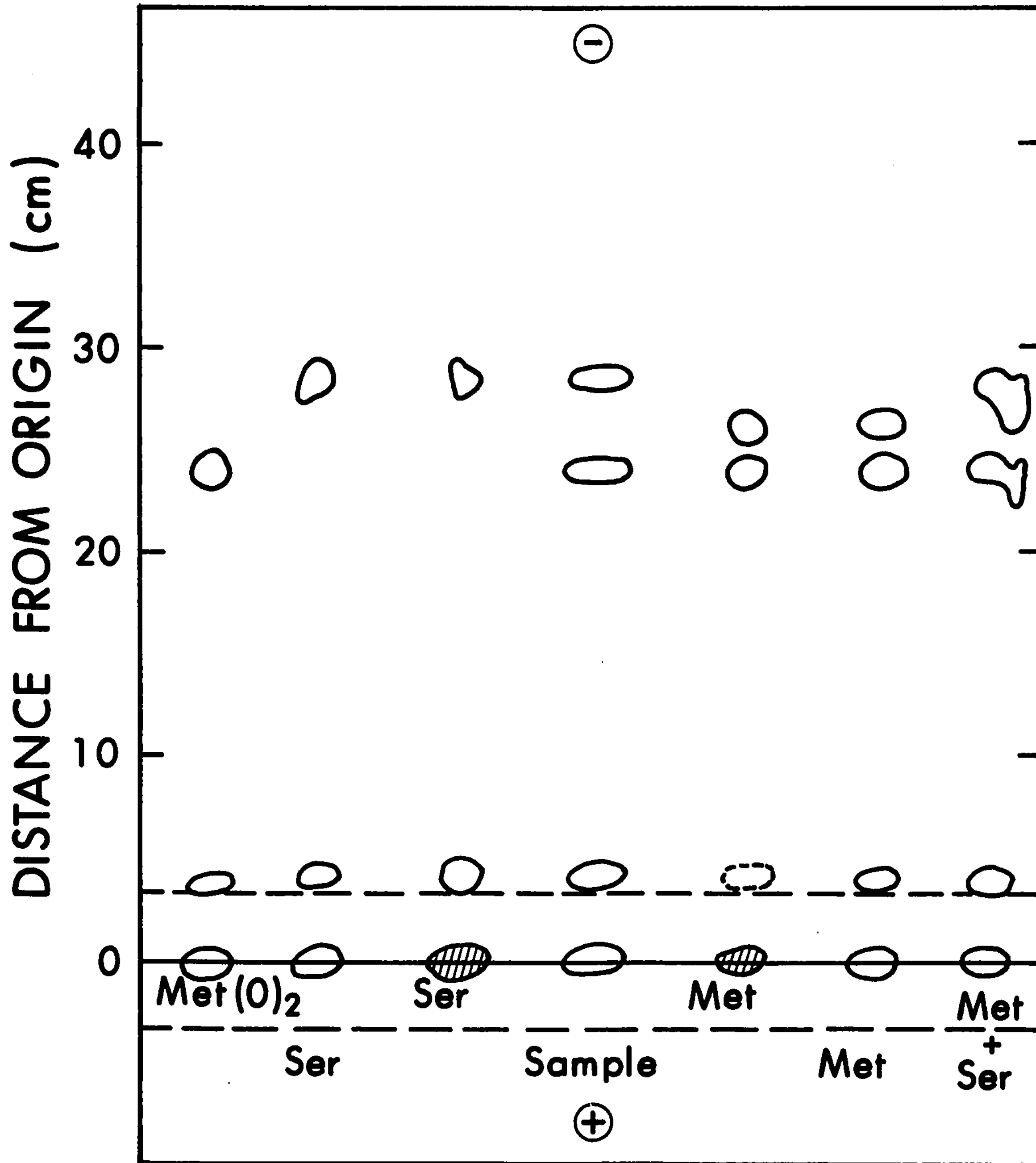
The separation of subunits is very difficult by column chromatography in the presence of denaturing reagents (32). Bridger (33) obtained two subunits in the SDS-gel electrophoresis run. The separation of subunits is essentially according to his method, following with elution of protein from gels as described by Weber and Osborn (45). Pure enzyme (1.5 mg) was incubated with SDS for 3 hours at 37°C and then subjected to electrophoresis on 10% polyacrylamide in the presence of SDS, with approximately 0.1 mg on each gel. One of the gels was stained and destained in order to localize the protein bands; the other 10 gels were cut and eluted twice with 0.1 per cent SDS solution. The two protein components, the α and the β subunits, were lyophilized. The α and β subunits were then dansylated and the N-terminal amino acid were identified by thin layer chromatography as described in above section A (i).

III. Results

A. Qualitative Determination of N-terminal Residues

Dansylated amino acid derivatives identified by thin layer chromatography were DNS-Met, DNS-Met-sulfoxide, DNS-Ser and DNS-basic amino acids. The DNS-basic amino acids may have consisted of α-DNS-His, ε-DNS-Lys, α-DNS-Lys and/or DNS-Arg. The DNS-Met-sulfoxide is an oxidized derivative of DNS-Met. The positions of DNS-Ser and DNS-Met-sulfone are very close on the plate. In order to confirm that we have DNS-Met and DNS-Ser in the N-terminal positions of SCS, the hydrolysates of DNS-protein were applied to Whatman No. 3 MM paper and run on flat-plate electrophoresis at pH 4.38 (6 KV, 2.5 hr) with standard marker DNS-Met, DNS-Met (O), DNS-Met $(O)_2$ and DNS-Ser on either side. The DNS-Ser, DNS-Met and DNS-OH were not clearly spearated at this pH run. The area containing substantial DNS-fluorescence near DNS-OH was cut out and sewed on another No. 3 MM paper, with standard DNS-amino acid also applied as markers as before. The paper was subjected to electrophoresis at pH 1.8 (3 KV, 80 min) in the tank. This gave a good separation, but we feared that electrophoresis of the sample across the sewing area and the presence of DNS-OH might affect the results. Therefore we cut out the positions of DNS-Ser and DNS-Met, including the standard, and sewed these to a new paper and subjected them to pH 1.8 electrophoresis with new standard markers in between. The results, shown in Figure 8, clearly demonstrate the presence of DNS-Met and DNS-Ser in the hydrolysate of dansylated protein. DNS-Met gradually converts to

FIGURE 8: Electrophoretic mobilities of dansyl-amino acids at pH 1.8 (3 KV, 80 min). Shadowed areas indicate new standard dansyl amino acid. Shown at the origin are the first applied sample and standard DNS-amino acids. The broken lines indicate the sewing area. See text for details.


⊖
DISTANCE FROM ORIGIN (cm)
40
30
20
10
0
Met(O)2
Ser
Ser
Sample
Met
Met
Met
+
Ser
⊕

DNS-Met (O) and DNS-Met $(O)_2$ during the electrophoresis run as seen from the behaviour of the standards. The phosphorylated protein also gave the same result, with DNS-Met and DNS-Ser. These experiments show clearly that SCS has both methionine and serine in the N-terminal position.

B. Quantitative Determination of N-terminal Residues

Fraction A (p. 39) contains neutral and acidic amino acids: methionine, serine, glutamic acid, and a slight trace of glycine and of alanine. The glutamic acid is derived from pyrrolidone carboxylic acid and pyrrolidone carboxyl peptide. The amount of protein in the N-terminal analysis can be determined from a quantitative amino acid analysis of the carbamyl protein based upon the number of leucine or alanine residues in the protein (see Chapter VI). The quantitative molar ratio of methionine to protein is 2.0. This means that 1 μmole of SCS gives 2 μmole of methionine, in keeping with the proposed $\alpha_2\beta_2$ structure. However, the ratio of serine to the protein is only 0.45. Nevertheless, owing to the instability of the hydantoin of serine in acid hydrolysis, the recovery of serine is only 20% (62). Therefore, the ratio of serine to the protein is about 2.2 if the correction factor is added. A trace amount of glycine may come from degradation of serine. The fractions B, C-1 and C-2 show only slight traces of lysine, histidine and arginine. These results rule out basic amino acid in the N-terminal of SCS. These experiments lend further support to the proposal (33) that SCS has an $\alpha_2\beta_2$ type of subunit structure.

C. N-terminal Residues of Isolated Subunits

The only α-dansylated amino acid identified by polyamide thin layer chromatography of the hydrolysate of the dansylated α subunit was serine. The dansylated amino acid found in the plate corresponding to the β-subunit was DNS-Met, and a trace of DNS-basic amino acids which derive from their side chains. The reason why DNS-Met (0) did not appear on the plate at this time may be due to the presence of 2-mercaptoethanol in the electrophoresis run. Therefore, the N-terminal of non-phosphorylated β subunit of SCS is methionine, and that of the phosphorylated α subunit is serine. This excludes the possibility that the N-terminal of methionine of SCS is the same methionine residue as that found in the N-terminal position of the active site peptide containing the phosphohistidine residue (see Chapter IV).

IV. Discussion

The results establish that SCS as isolated from succinate-grown E. coli is composed of two species of subunits, with the phosphorylated one bearing the serine and the non-phosphorylated subunit having methionine at its N-terminals. The quantitative analysis of N-terminal reveals that SCS has about two moles each of these N-terminal residues per mole of enzyme. The data are clearly consistent with the existence of equimolar amounts of the two subunit species in the native enzyme, as suggested by Bridger (33), with an $\alpha_2\beta_2$ type of subunit structure of overall molecular weight near 137,000 daltons. These experiments provide an explanation for the observation that the enzyme can be phosphorylated by more than one and approaching two phosphoryl groups per mole of enzyme. The reason why the phosphorylation of the second α subunit is more difficult

than the first is still not clear. One possibility may be due to undetected heterogeneity in the α subunit. A more likely explanation may involve some kind of anti-cooperativity as suggested by Bridger (33).

Benson *et al*. (78) described a slow irreversible succinylation of the enzyme following incubation with succinyl CoA and P_i. Bridger (64) succinylated SCS by their method, and found 2.5 succinate moieties bound per 140,000 daltons of the enzyme. Most of H^3-succinate was found in the β-subunit following SDS gel electrophoresis. This provides additional support for the $\alpha_2\beta_2$ structure, and mildly suggests that some portion of the active site may be composed with polypeptide from the β-subunit, which does not bear the phosphohistidine residue.

CHAPTER VI

AMINO ACID COMPOSITION AND PEPTIDE MAPPING OF SCS

I. Introduction

SCS of *E. coli* is composed of a number of polypeptide chains (32,33). It is imperative that both the number and kinds of subunits be established before extensive chemical studies are begun. In this Chapter, we describe experiments to investigate the subunit structure from a different point of view. A common method of distinguishing between identical and different polypeptide chains in a protein is by treatment with the proteolytic enzyme trypsin. If the number of lysine and arginine residues in the protein is known, the number of peptides obtained by tryptic digestion is predictable. That is, a protein that is composed of identical subunits will give rise to fewer different peptides than if the subunits differ. The previous Chapter dealt with qualitative and quantitative determination of the N-terminal residues of SCS. Qualitative determination gave an indication of the number of different kinds of subunits and the quantitative determination allowed us to calculate the number of subunits per molecule, even if the subunits were identical. Amino acid composition and tryptic mapping of SCS are reported in this Chapter. The results of Chapter V and Chapter VI all confirm that SCS from *E. coli* has an $\alpha_2\beta_2$ type of subunit structure.

II. Methods

A. Amino Acid Analysis

The amino acid composition studies of SCS were performed as described in Chapter II, Section II, C (ii). The subunits of SCS were isolated as described in Chapter V, II, B, with 0.1 per cent SDS as the gel eluent. Solutions of each of the α and β subunit were divided into six parts and then subjected to SDS removal by the method of Weber and Osborn (45) which makes use of cold acetone. The protein was hydrolyzed and analyses were performed as described in Chapter II, IIC (ii).

B. Trypsin Digestion and Peptide Mapping

Approximately 8 mg of SCS was oxidized with performic acid by the method of Hirs (65). The performate-oxidized protein in 5 ml of 0.05 M N-ethylmorpholine-acetic acid buffer, pH 8.0 was digested with 160 μg of TPCK-trypsin at 37°C for 5 hours. The peptides were separated by a two-dimensional electrophoresis technique at pH 6.5 and pH 1.8 as follows: the mixture was lyophilized, dissolved in a small amount of distilled water, and applied to Whatman No. 3 MM filter paper and subjected to electrophoresis at pH 6.5 buffer (acetic acid-pyridine-water) at 3 KV for 40 minutes. Strips of acidic and basic peptide were divided into two and cut out, each sewn on a sheet of No. 3 MM paper, and the second dimension electrophoresis was carried out at pH 1.8 (acetic acid-formic acid-water), 3KV for 40 minutes. The section of the 6.5 electrophoretogram containing the neutral peptides was cut out, sewn on a sheet of No. 3 MM paper, and resubjected to electrophoresis in the first dimension at pH 6.5, 3 KV for 2 hours, cut out into two

portions, and each was subjected to electrophoresis in the second dimension at pH 1.8, 3 KV for 40 minutes. One of two air-dried papers was dipped in cadmium-ninhydrin reagent of Heilmann et al. (50), and the color was allowed to develop in an oven. The other one was stained with Pauly reagent for histidine containing peptides.

III. Results

A. Amino Acid Analysis

The amino acid composition of the purified SCS from E. coli is shown in Table V. The first column gives the data of Leitzmann et al. (32) which were published in 1970 subsequent to our determination and were based on a M.W. of 146,000. The second column are our data based upon a M.W. of 140,000, which are very similar to the analysis of Leitzmann et al. The high content of the acidic amino acid is in harmony with the pI value of 5.5 of SCS which determined by Leitzmann et al. (32) with an isoelectric focusing experiment. Also listed in the Table are the amino acid compositions of the purified subunits, assuming a M.W. for the α-subunit of 30,000 and for the β-chain of 40,000 as estimated by SDS-gel electrophoresis (33). All of the data give the moles of amino acid per mole of the given protein species, calculated on the assumption that the enzyme contained only amino acids. The expected data for the $\alpha_2\beta_2$ and also shown on the Table are remarkably consistent with the amino acid composition of whole protein, thus providing still more convincing evidence for the $\alpha_2\beta_2$ subunit structure.

TABLE V

AMINO ACID COMPOSITION OF *E. COLI* SCS AND ITS CONSTITUENT SUBUNITS

Amino Acid	Composition				
	(1)	(2)	α subunit	β subunit	$\alpha_2\beta_2$
Lysine	90.5	90.6±1.8	17.7	21.2	78
Histidine	22.2	19.7±0.5	6.5	4.6	22
Arginine	41.1	44.1±0.5	5.0	15.5	42
Aspartic acid	111.2	107.8±0.9	16.7	37.5	108
Threonine	84.0	80.2	24.9	14.8	80
Serine	43.0	40.3	14.9	11.6	52
Glutamic acid	147.7	139.2±0.8	26.0	43.8	140
Proline	58.4	55.4	12.7	14.7	56
Glycine	172.6	164±1.5	41.0	44.5	170
Alanine	159.2	147.8±1.5	29.1	45.4	148
Half Cystine	26.7	24.4	n.d.	n.d.	n.d.
Valine	126.2	119.0	23.0	37.9	122
Methionine	27.6	27.5±0.8	6.2	8.3	28
Isoleucine	99.9	96.8±1.5	27.9	22.2	100
Leucine	102.2	106.3±0.3	15.2	38.3	106
Tyrosine	25.8	22.8±0.3	3.7	3.9	16
Phenylalanine	39.0	39.6±0.2	5.9	7.3	28
Tryptophan	8.2	8.5	n.d.	n.d.	n.d.

(1) Data taken from Leitzmann *et al*. (32).

(2) Data from our laboratory.

n.d. not determined.

B. Peptide Mapping of SCS

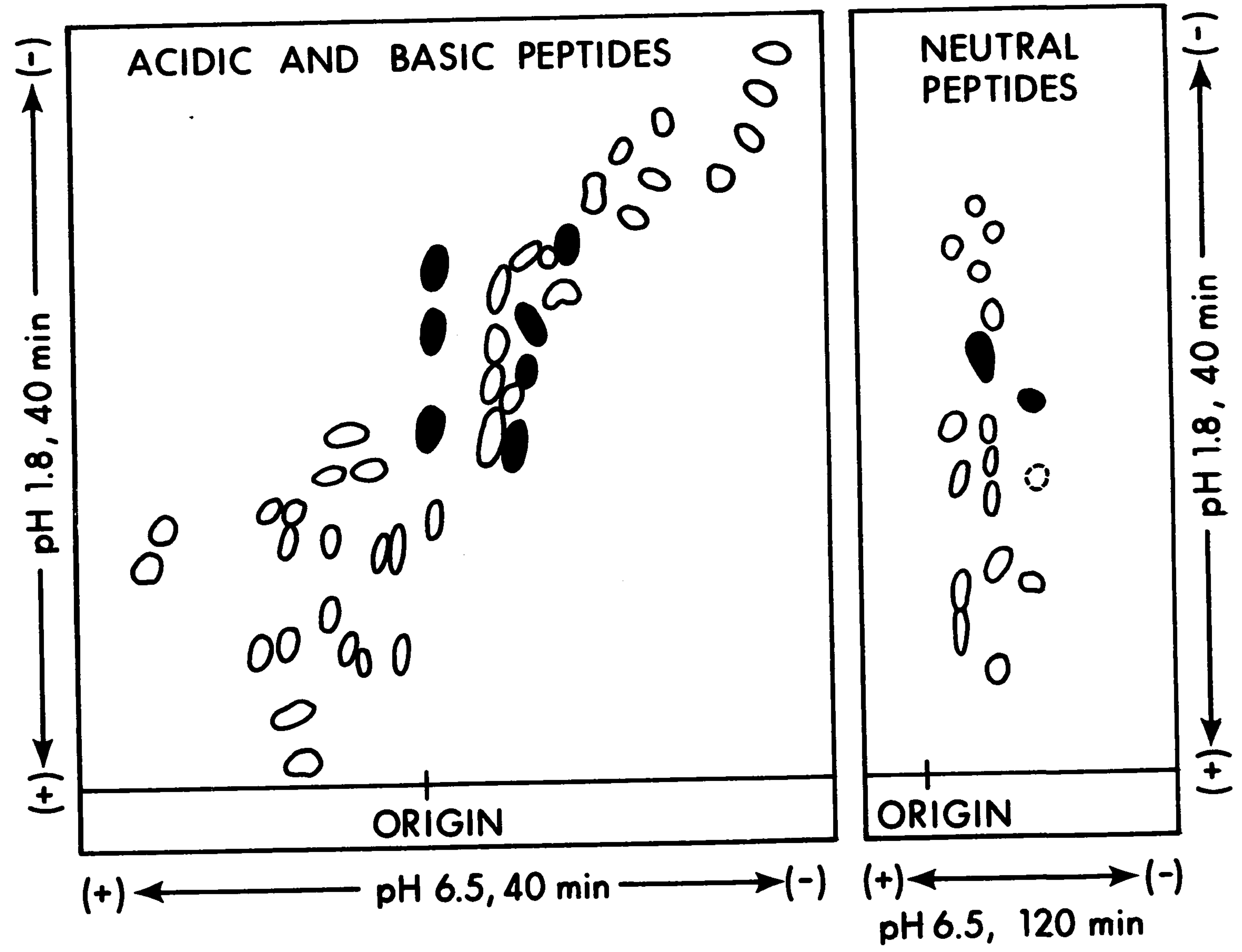
The peptide map of SCS from *E. coli* is shown on Figure 9, with an estimated total of 61 ninhydrin-positive spots and 9 histidine containing peptides. Amino acid analyses given in Table V showed the presence of 90 lysine residues and 41 - 44 arginine residues per mole of SCS. As mentioned before in Chapter IV, peptide H-3 contains two histidine residues side by side, and was obtained by treating with TPCK-trypsin. These data are therefore consistent with the subunit composition $\alpha_2\beta_2$ of SCS, since the observed peptide maps indicate a unique amino acid sequence of molecular weight near 70,000.

IV. Discussion

The results of amino acid composition and peptide mapping studies clearly strengthen the conclusion of Chapter V of this dissertation that SCS has an $\alpha_2\beta_2$ type of subunit composition. The structure-function relationship of two non-identical subunits of the *E. coli* SCS is not known. One possibility is that both subunits have a catalytic function with α subunits catalyzing phosphorylation of reaction (1) and β subunit binding with succinate by reaction (2) in the Chapter I.

It is interesting to note that SCS of pig heart, in contrast to the *E. coli* SCS, appears to be constituted as an αβ dimer of two non-identical polypeptides of overall M.W. about 78,000, with the smaller subunit (α) undergoing phosphorylation by GTP (26). It seems that *E. coli* SCS may correspond to a larger assembly of two such αβ dimers. The dissociation of *E. coli* SCS into the dimer (αβ) and its catalytic ability will be interesting to further studies.

FIGURE 9: Peptide maps obtained with TPCK-tryptic digests of performic acid-oxidized SCS. The samples were subjected to two dimensional electrophoresis as described in Methods. The solid spots gave a positive test for histidine by the Pauly reagent.


ACIDIC AND BASIC PEPTIDES
NEUTRAL PEPTIDES
ORIGIN
ORIGIN
(+) pH 6.5, 40 min (-)
(+) (-)
pH 6.5, 120 min
(+) pH 1.8, 40 min (-)
(+) pH 1.8, 40 min (-)

CHAPTER VII

CONFORMATIONAL CHANGES IN SCS

I. Introduction

The digestion of the enzyme by limited proteolysis (75) has proved to be a sensitive tool for the detection of subtle modification of protein structure manifested by changes in the environment of proteolytically susceptible bonds, as exemplified by such studies with ribonuclease (66), hemoglobin (67), serum albumin (68), aspartate transcarbamylase (69) and glyceraldehyde-3-phosphate dehydrogenase (70). In the case of aspartate transcarbamylase, McClintock and Markus (71) found that the rate of tryptic digestion of the enzyme is significantly increased when the substrate, aspartate, is present in the digestion mixture. On the other hand, both ATP and CTP, allosteric activator and inhibitor, respectively, cause a marked decrease in digestibility of the enzyme. They also indicated that the primary target of tryptic attack is the regulatory subunit, and that when both ATP and CTP are present in the digestion, they protect against the loss of allosteric properties.

In the case of E. coli succinyl CoA synthetase, we wished to apply this technique to a study of substrate-induced conformational changes (72). In particular, we desired a possible explanation for the "half-the-sites-reactivity" (76) exhibited by SCS, whereby the phosphorylation of the first α-subunit of the oligomeric protein is achieved with ease, while phosphorylation of the second, apparently

equivalent site, is much more unfavorable. A possible explanation for this is provided by experiments which are described in this Chapter.

While in impure preparations the effects of substrates can only be measured by following changes in enzyme activity, the high purity of SCS in the present study permitted the correlation of changes in enzyme activity with the degree of enzyme breakdown. The results reported in this Chapter show that this technique can be profitably applied to the study of conformational changes in terms of the induced-fit model of Koshland (72) with phosphorylation of this enzyme apparently promoting a rather large change in its three-dimensional structure.

II. Methods

A. Enzyme Concentration and Activity Assays

Enzyme activity was measured by appearance of absorbance at 230 nm, which accompanies succinyl-CoA formation, at 25°C with the aid of a Cary model 15 spectrophotometer. Enzyme concentrations were estimated from the A_{280}, based upon the extinction coefficient $A_{280}^{0.1\%}$ = 0.511 given by Ramaley et al. (22).

B. Dephosphorylation of SCS

All the enzyme used for trypsin inactivation studies was dephosphorylated previous to the experiment. The dephosphorylation step was carried out essentially according to the method of Ramaley et al. (22), with the reaction mixture containing SCS 10 mg, 10 mM Mg^{++}, 1 mM ADP, hexokinase (50 µg in 5 µℓ $(NH_4)_2SO_4$ suspension) and 0.05 M glucose in 0.1 M

Tris-Cl, pH 7.5. The reaction was allowed to proceed for 20 minutes at 25°C, 0.1 ml of 0.1 M EDTA solution was added to stop the reaction, and the solution was cooled and passed through a G-50 Sephadex column (1.5 x 60 cm) and eluted with the same buffer. The dephosphorylated enzyme was used throughout the entire studies without testing the phosphoenzyme remaining.

Zero time activities were determined before the reactions were initiated by the addition of protease.

III. Results

A. Effect of Substrates on Susceptibility of SCS to Trypsin

The effects of various substrates on the rate of trypsin-catalyzed inactivation of SCS are shown in Figure 10. ATP is seen to protect the enzyme, while succinate increases trypsin digestibility. The effect of CoA is small while the presence of all substrates significantly enhances digestibility to trypsin. The control, in the absence of added substrates, shows a biphasic curve. According to Moffet's observation that the phosphorylated enzyme is much more stable to trypsin than the dephosphorylated species, this may indicate some degree of residual phosphorylation (73). The reason for ATP protection was later found by Moffet (73) to be directly related to phosphorylation of the enzyme. ATP is known to phosphorylate SCS (22), while succinate is capable of bringing about the dephosphorylation of the enzyme (63). Moffet was able to show that succinate will dephosphorylate SCS in the presence of Mg^{++} ion and so increase the digestibility of the enzyme, and that in this case as well there was a direct relationship between

the degree of phosphorylation and the susceptibility to trypsin. Thus the degree of phosphorylation of the enzyme might explain all the results of Figure 10. A possible rationale for a significant fraction of enzyme remaining in the phosphorylated form for the experiment of Figure 10 may relate to the stability of these forms of enzyme. The enzyme was stored for half a day between dephosphorylation and the experiment. We have found that the dephosphorylated form of SCS will lose activity quickly on storage, whereas the phosphorylated species is much more stable. Moffet, in his thesis (73), reported that the dephosphorylated SCS will lose half of the enzyme activity in 1.2 days, compared to a half life of 100 days for E-P. The instability of dephosphorylated SCS was investigated further and the results are also reported later in this Chapter. It is quite conceivable that only a small fraction of enzyme was not dephosphorylated, but that this then came to represent a significant fraction of the enzyme activity owing to the loss of activity of the dephosphorylated fraction on storage.

It is important to point out that either the presence of the substrate ATP or covalent phosphorylation of the enzyme tremendously protects it from digestion by trypsin. Figure 11 shows the protective effects of the presence of different concentrations of ATP in the presence of EDTA and absence of added Mg^{++}. Although the original interpretation was that covalent phosphorylation could not account for the protection in the absence of Mg^{++}, it was subsequently shown that the enzyme will undergo slow phosphorylation under these conditions (73). We also found that ATP will give the same degree of protection even though either substrate, succinate or CoA, is present. This result confirms that the rate of dephosphorylation by succinate is slow and has

FIGURE 10: Effect of trypsin on the activity of SCS in the presence and absence of substrates. The reaction mixture contained 0.54 mg /ml SCS (S.A. = 10.2), 100 mM Tris-Cl, pH 7.4, 10 mM $MgCl_2$, 0.0004% trypsin and additions as shown. Concentrations of added substrates: succinate, 10 mM; ATP, 0.5 mM; CoA, 0.2 mM.

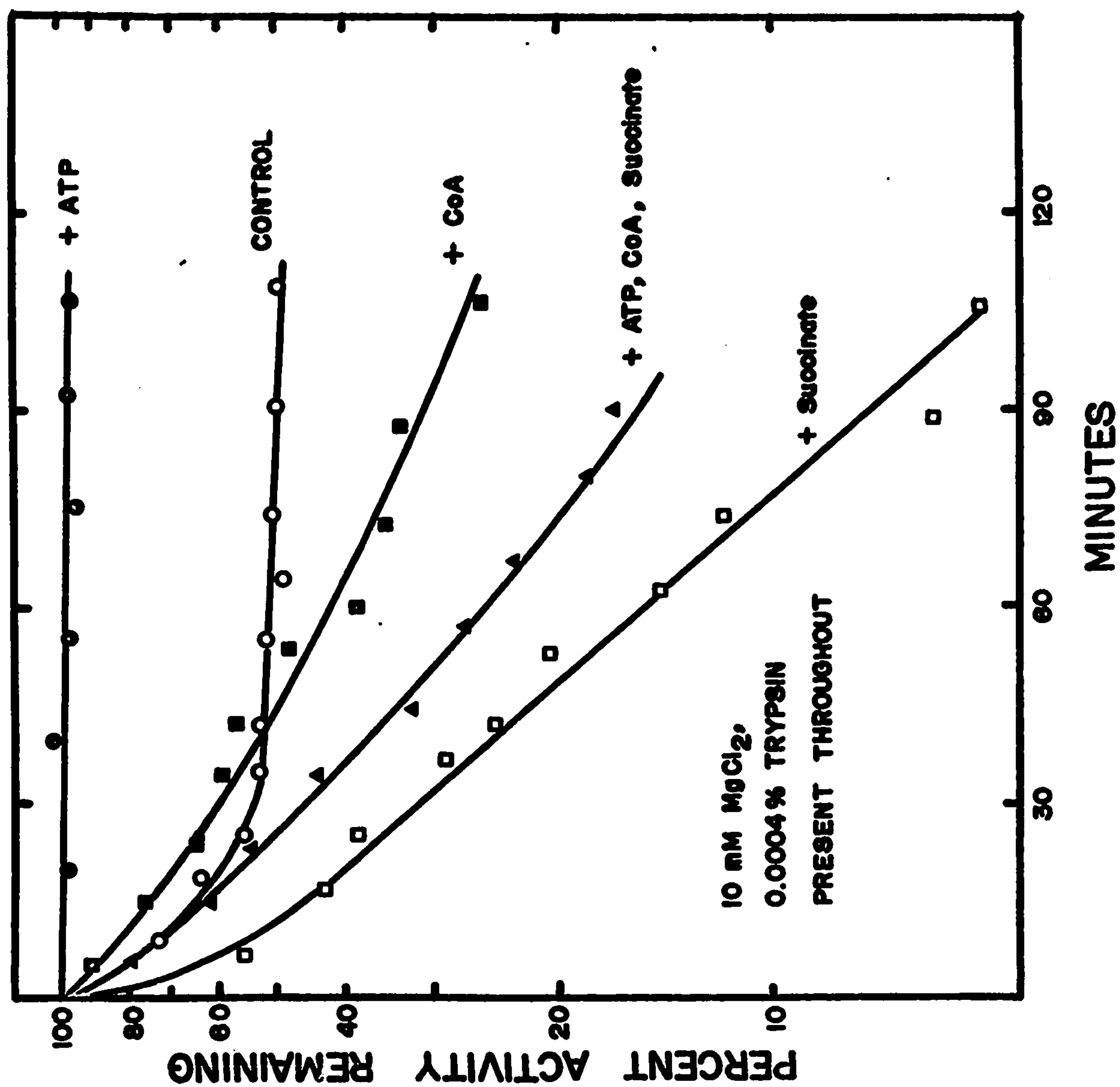
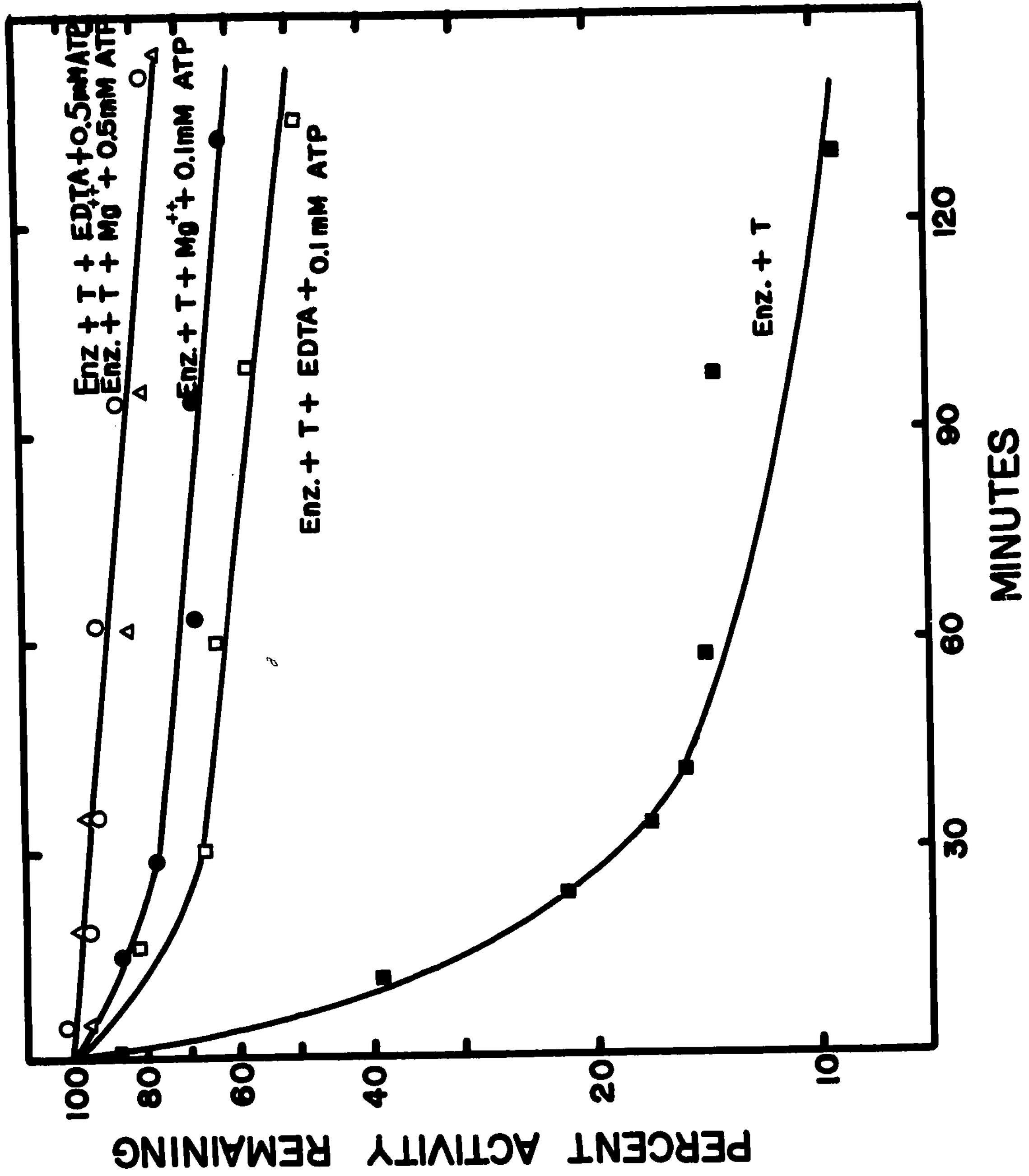

+ ATP
CONTROL
+ CoA
+ ATP, CoA, Succinate
+ Succinate
10 mM MgCl2,
0.0004 % TRYPSIN
PRESENT THROUGHOUT
PERCENT ACTIVITY REMAINING
100
80
60
40
20
10
30
60
90
120
MINUTES

FIGURE 11. Inactivation of SCS by trypsin in the presence of ATP and in the presence and absence of EDTA. Reaction mixture contained 0.4 mg /ml SCS (S.A. = 6.0), 0.1 M Tris-Cl, pH 7.5, 0.0006% trypsin and other additions as shown. Concentration of added reagents: $MgCl_2$, 10 mM; EDTA, 10 mM. T represents trypsin.



Enz + T + EDTA + 0.5mM ATP
Enz. + T + Mg++ + 0.5mM ATP
Enz. + T + Mg++ + 0.1mM ATP
Enz. + T + EDTA + 0.1mM ATP
Enz. + T
PERCENT ACTIVITY REMAINING
100
80
60
40
20
10
MINUTES
30
60
90
120

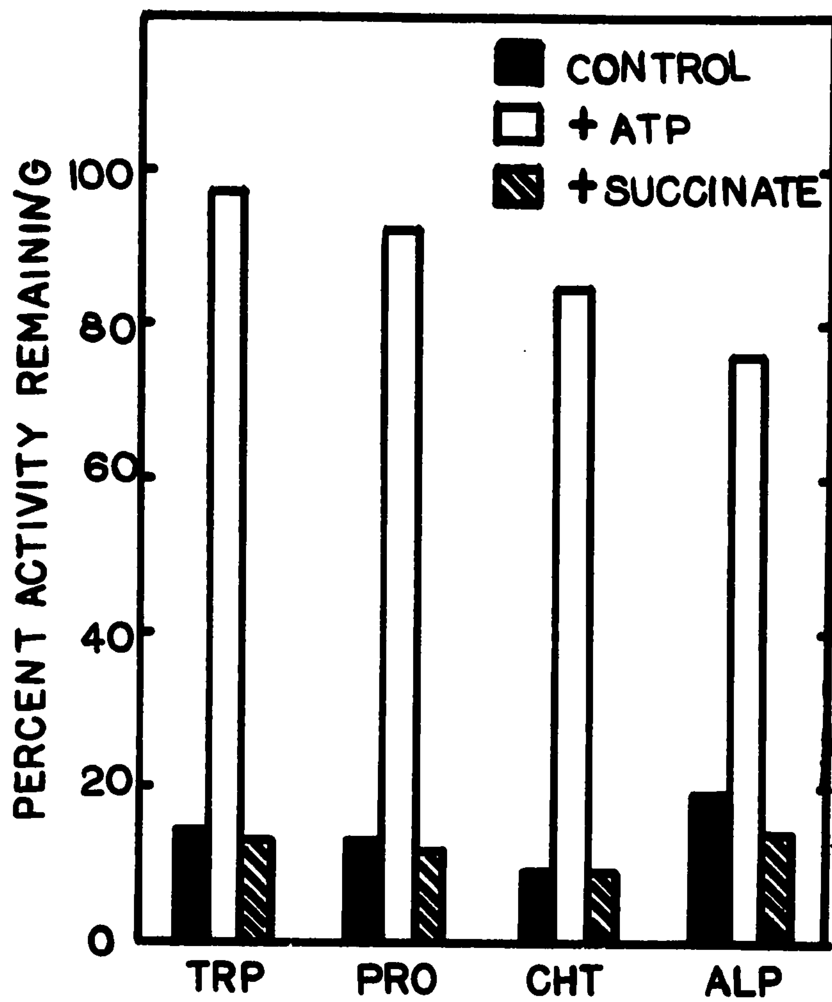
no effect within a two hour incubation with ATP. The result is also consistent with the kinetic mechanism (28) which has a preferred pathway involving ATP binding to the free enzyme.

B. Comparison of Digestibility by Several Proteases

The effects of the substrates were also studied with three additional proteases of differing specificities. Figure 12 shows that the presence of ATP will cause a substantial decrease in the sensitivity of SCS to each of the proteases. The results obtained with TPCK-trypsin, pronase, TLCK-α-chymotrypsin and α-lytic protease were quite similar. The dephosphorylated enzyme used for this experiment was freshly prepared. Succinate has little effect on the amount of residual activity at the selected time. Particularly significant is the finding that the presence of ATP and subsequent phosphorylation of SCS depresses thedigestibility when tested by any of the four enzymes. It would be improbable that a single nucleotide or phosphoryl group could shield each of the different peptide bonds required for action by each of the proteases used. The general agreement of the results obtained with the different proteases therefore indicates that the observed protective effect is due to conformational changes rather than to steric shielding of susceptible bonds. These results then lend additional support to the interpretation given to the experiment carried out with TPCK-trypsin alone.

After the measurement of residual activity after TPCK-trypsin digestion in the experiment of Figure 12, 10 μℓ of 2-mercaptoethanol and 50 λ of 10% SDS in 0.01 M phosphate buffer, pH 7.2 were added, and the reaction mixture was then dipped into a boiling water for 3 minutes in

FIGURE 12: Effects of ATP and succinate on rate of inactivation of succinyl CoA synthetase by a variety of proteolytic enzymes. In each case, the fraction of activity remaining was determined following exposure of succinyl CoA synthetase to the protease in the presence of 0.5 mM ATP or of 10 mM succinate, and in controls with no addition of substrates. TRP: TPCK-treated trypsin (0.004 mg/ml), reaction time 50 min; PRO: Pronase (Calbiochem. Grade A, 0.025 mg/ml), reaction time 10 min; CHT: TLCK-treated α-chymotrypsin (0.04 mg/ml) reaction time 30 min; ALP: α-lytic protease of _Sorangium_ sp. (36) (0.04 mg/ml), reaction time 85 min. The enzyme was dephosphorylated prior to the experiment.


CONTROL
+ ATP
+ SUCCINATE
PERCENT ACTIVITY REMAINING
100
80
60
40
20
0
TRP
PRO
CHT
ALP

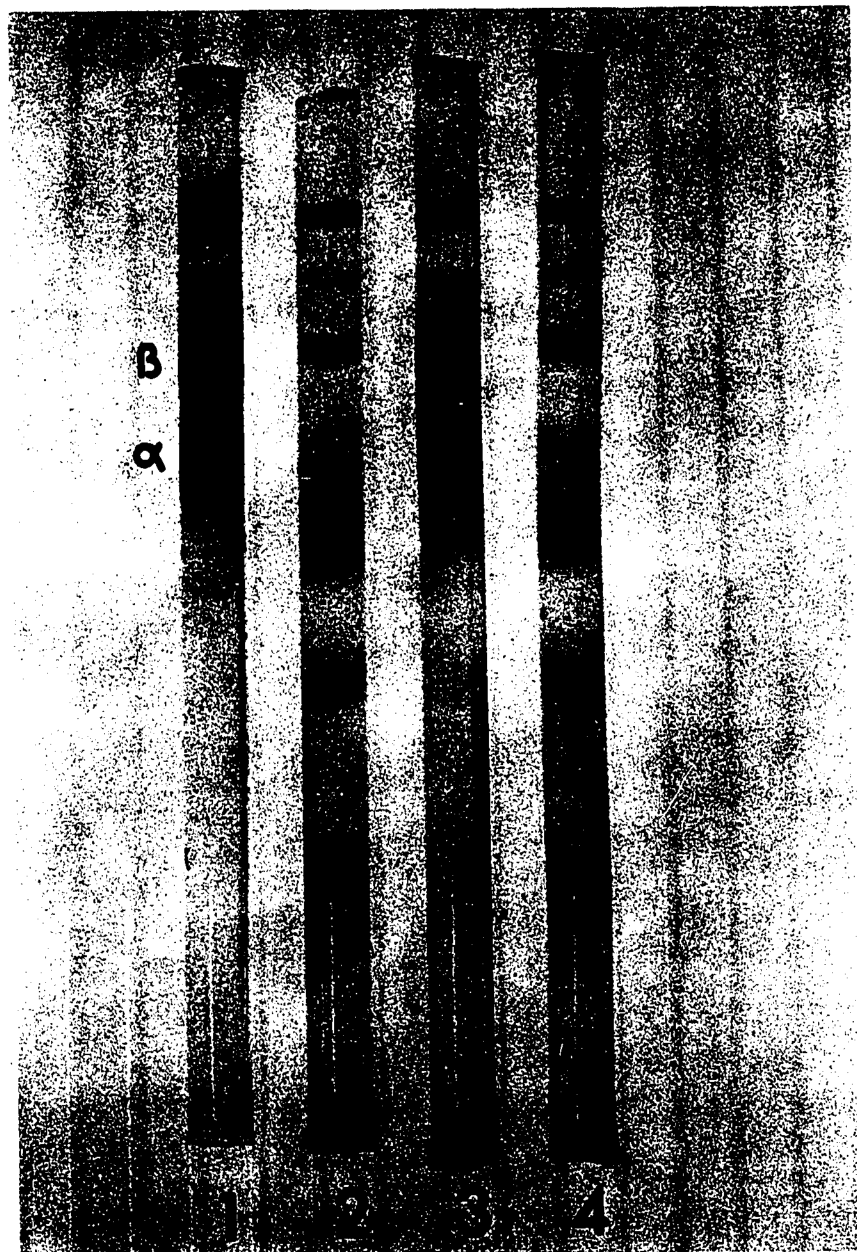
order to inactivate trypsin. The reaction mixture was then incubated at 37°C for 2 hours and SDS-gel electrophoresis was carried out as described in Chapter II.

Figure 13 shows the distribution of the digested enzyme in the SDS gel after treating with TPCK-trypsin for 50 minutes. The amount of intact subunit apparent in the gel corresponds, roughly at least, to the residual enzyme activity shown on Figure 12. ATP seems to protect not only the α subunit but also the β subunit, as seen by gel 3. SCS is phosphorylated by ATP only in the α subunit, and yet such phosphorylation appears to induce a more stable conformation in both subunit species. If we compare the appearance of the α and β subunits in gel 1 and 2, it appears that the α subunit is more sensitive to proteolysis than the β subunit. These results are consistent with a substantial conformational change in the SCS molecule upon phosphorylation of the active site histidine residue by ATP.

C. <u>Dephosphorylation</u>

As we have mentioned before, phosphorylated and dephosphorylated SCS are quite different in their stability properties. Phosphorylated SCS is very stable even on treatment with protease, while dephosphorylated enzyme is extremely unstable during storage in the refrigerator. Figure 14 (A) and (B) give the specific activity of both two forms of the enzyme during storage at 4°C. We also examined the protein subunits by SDS gel electrophoresis after storage at 4°C for 1 month in the case of the sample of Figure 14 (A), and the results of this experiment are shown in Figure 15. All the samples of dephosphorylated enzyme show two bands in the standard gel studies (in the absence of SDS)

FIGURE 13: SDS-polyacrylamide gel electrophoresis patterns of succinyl CoA synthetase following exposure to trypsin. Electrophoresis was conducted in 10% polyacrylamide in the presence of SDS and 2-mercaptoethanol according to the method of Weber and Osborn (45). The dephosphorylated enzyme was incubated with TPCK-trypsin (0.004 mg/ml) for 50 min at 25° in the presence of 10 mM $MgCl_2$, 50 mM tris-HCl, 50 mM KCl, pH 7.2 with the following additions or exceptions: 1, enzyme without exposure to trypsin; 2, none; 3, 0.5 mM ATP; 4, 10 mM succinate. (The sharp bands at the top of the gels represent minor impurities in the enzyme preparation or the undissociated enzyme). Each gel is loaded with about 45 μg of enzyme.


B
α
1
2
3
4

as gel 3 of Figure 15, whereas the phosphorylated form is one band throughout the entire storage. The sedimentation velocity Schlieren pattern of the dephosphorylated sample shows only one peak with S_{obs} = 5.0S. Electrophoresis of the aged dephosphorylated enzyme in the presence of SDS (gel 5 of Figure 15) gives results which are reconciled with the above observation, with two bands close together and smaller than either of the intact subunits in size. It therefore appears that the activity loss of dephosphorylated enzyme is accompanied by attack by some contaminating proteases during the storage. The phosphoenzyme in gel 4 of Figure 15 clearly gives rise to two subunits, α and β, as found for fresh enzyme. We have examined the dephosphoenzyme after only one week of storage (the sample of Figure 14(B)) by means of SDS-gel electrophoresis; at that time the specific activity is 0.5 unit/mg. The results of Figure 16 also show the same trend as that seen in Figure 15, that the β subunit is apparently breaking down. The exact mechanism of the degradation of the dephosphorylated enzyme is not yet clearly understood, but from Figures 14, 15, and 16, the loss of activity is not likely due to the oxidation of SH-groups, but rather to the attack by contaminating proteases.

IV. Discussion

The results presented show that the presence of ATP will decrease the susceptibility of SCS to attack by TPCK-trypsin. Recently, the ability of saturating concentrations of inorganic phosphate to protect the enzyme from inactivation by trypsin was discovered by Moffet (73). He also indicated that the binding of

FIGURE 14: Decrease of specific activity of dephosphorylated and phosphorylated SCS upon storage at 4°C. ●–●, phosphorylated SCS 0.6 mg/ml; dephosphoenzyme, □–□ , 0.3 mg/ml; ◆–◆, 0.54 mg/ml; ▲–▲ , 1.2 mg/ml; ■–■ , 0.6 mg/ml; ○–○ , 0.86 mg/ml. B panel is the dephosphoenzyme used in the experiments of Fig. 12 and Fig. 13.

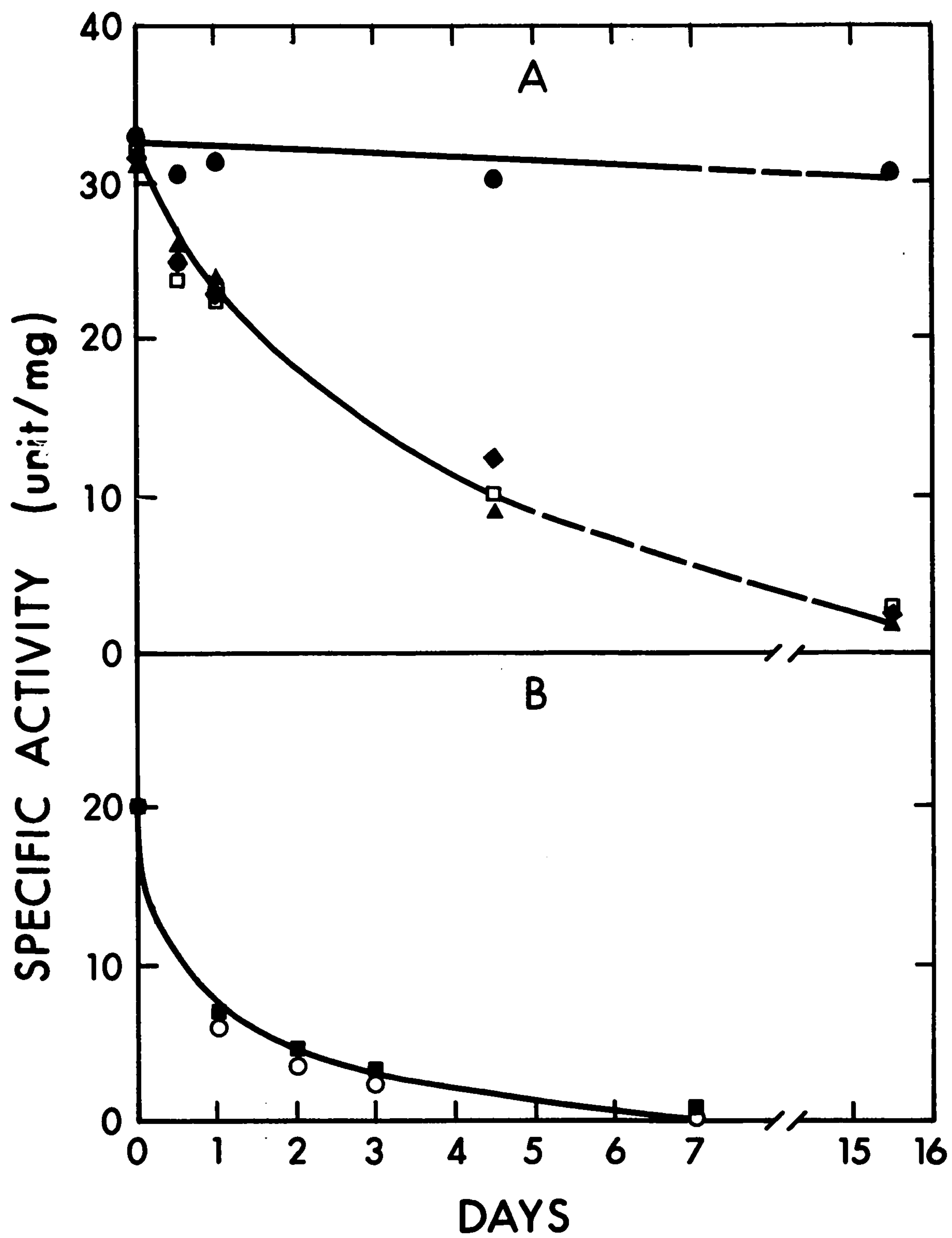

A
B
SPECIFIC ACTIVITY (unit/mg)
40
30
20
10
0
20
10
0
0
1
2
3
4
5
6
7
15
16
DAYS

FIGURE 15: Comparison of the gel electrophoretic patterns of sample from Fig. 14 (A) before and after storage at 4°C for one month.

1. 12 μg of one-month-aged phosphorylated SCS (sample of Fig. 14 (A) ●–●). The fresh sample shows one band.

2. 6 μg of fresh dephosphorylated SCS (sample of Fig. 14 (A) □–□). The other dephosphorylated enzymes shown in the Fig. 14 (A) also give rise to only one band.

3. 15 μg of one-month-aged dephosphorylated SCS (sample of Fig. 14 (A) □–□). The other dephosphorylated SCS shown in the Fig. 14 (A) also give rise to two bands.

4. 30 μg of one-month-aged ^{32}P-SCS shows two bands as is the case for the freshly prepared enzyme.

5. 30 μg of one-month-aged dephosphorylated SCS shows two bands closer together and of lower M.W. (sample from Fig. 14 (A) ▲–▲). The other dephosphorylated SCS also have the same patterns.

-SDS
+SDS
1
2
3
4
5

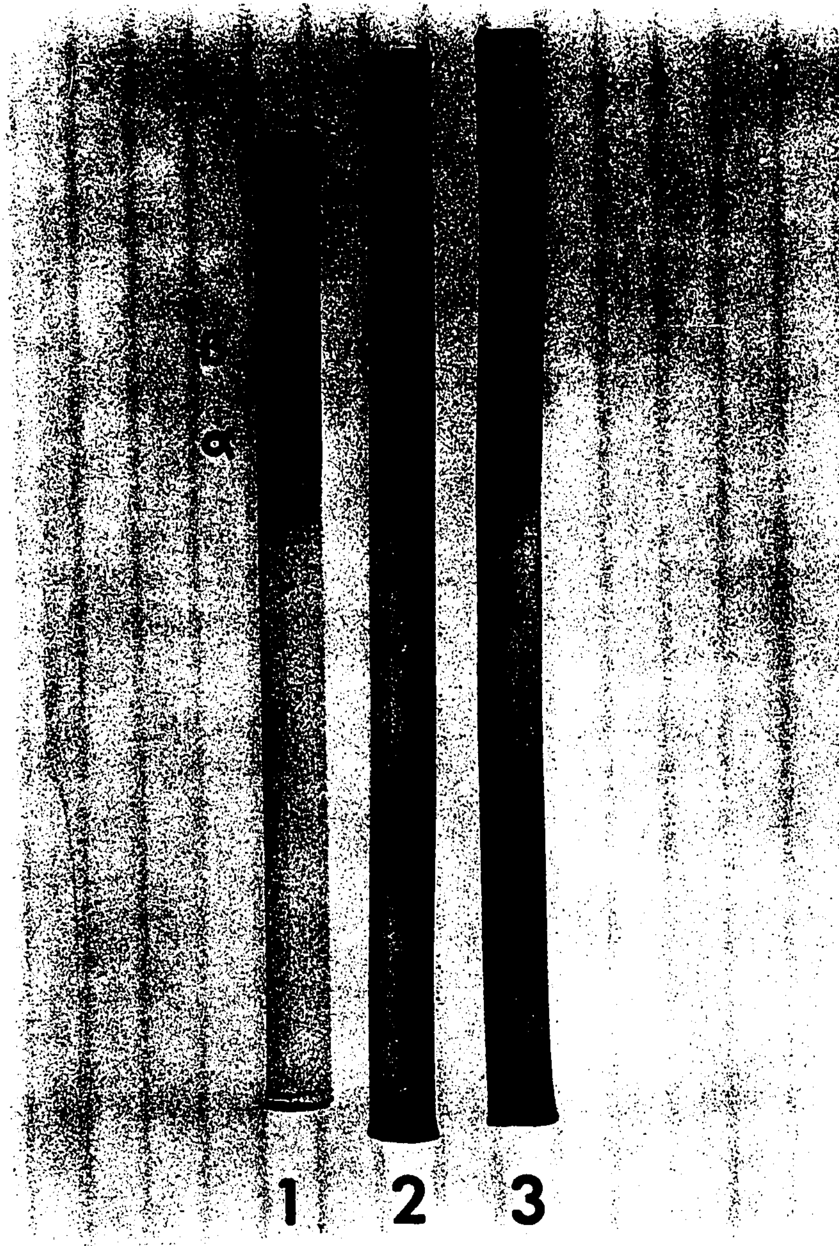
FIGURE 16: SDS-gel electrophoresis of sample Fig. 14 (B) after one week storage at 4°C.

1. 50 μg of fresh preparation of dephosphorylated SCS of Fig. 14 (B) O-O . Shows both α and β bands.

2. 24 μg of one-week-aged dephosphorylated SCS (Fig. 14 (B) ■-■ sample) shows β subunit to be decreasing in amount.

3. 25 μg of one-week-aged dephosphorylated SCS (Fig. 14 (B) O-O sample) also shows β subunit to be decreasing in amount.

(The sharp bands at the top of the gels represent minor impurities in the enzyme preparation).


1
2
3

phosphate would change the height of the emission peak of the fluorescent spectrum of the enzyme, and that a similar effect was observed following covalent phosphorylation.

It has been previously mentioned by Leitzmann et al. (32) that there is considerable variation in the catalytic activities of pure preparation from different laboratories and also within the same laboratory. The phosphorylation of the enzyme, or the presence of saturating concentrations of inorganic phosphate as ordinarily used in the enzyme preparation will stabilize the enzyme activity, whereas the dephosphorylated enzyme loses its activity very quickly, as seen in these experiments. It is probable that the degree of phosphorylation will contribute to the observed paradoxical variation of activities. It is certain that the enzyme is more stable in phosphate buffer than in Tris-Cl buffer.

Figure 14 shows that the rate of inactivation is not the same for two preparations, which may be explained by the amount of contamination by proteases in the solution. Introduction of contaminating proteases may occur during the procedure of dephosphorylation in which yeast hexokinase is used (p. 52). Pringle (79) pointed out that the commercial preparations of yeast hexokinase are contaminated with a trace of at least one proteolytic enzyme. In any case, the dephosphorylated enzyme is more unstable than the phosphorylated enzyme.

CHAPTER VIII

CONCLUSION

The kinetics of acid-catalyzed hydrolysis of phosphorylated SCS are similar to the model of phosphohistidine consistent with the formation of a phosphohistidine intermediate in the SCS reaction. Our data in Chapters III and IV exclude the possibility of some poor substrates which react chemically, nonspecifically, and sufficiently slowly to form other phosphorylated residues which are not intermediates, as the case of NDP kinase (11,17). The isolation of this phosphohistidine-containing peptide following treatment with TPCK-trypsin also clearly excludes the transfer of intermediates during alkaline hydrolysis such as has been proposed for the controversial enzyme ATP-citrate lyase (12,15). Our simple method of isolation of phosphohistidine-containing peptide may be of utility in studies of other enzymes. We also found that the N-terminal methionine of the active site phosphohistidine peptide (Table IV) is not the N-terminal of α subunit of SCS. The possible function of methionine near the active site region will be further studied.

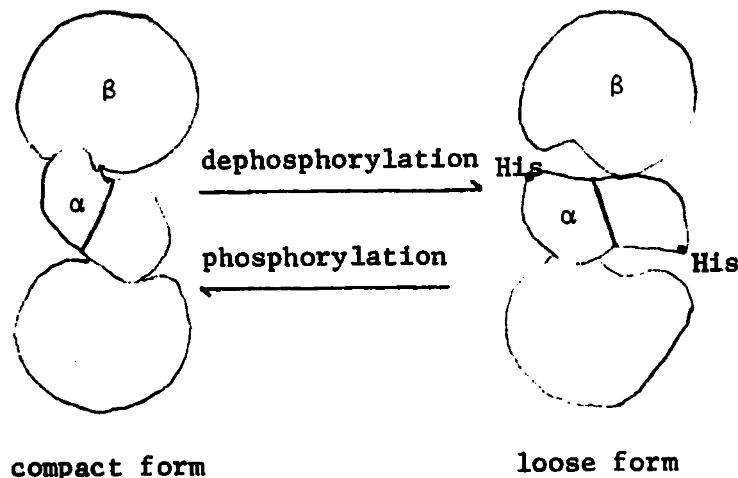
Theoretical considerations of the role of covalent intermediates in a catalysis suggest that the reactive amino acid on the enzyme must serve as a better attacking group than the final acceptor and as a better leaving group than the leaving group on the initial substrate donor (74). The phosphohistidine intermediate of SCS might serve as such a catalytic group as discussed above, might preserve the high

energy character of a bond in the multistage reaction and might provide a sterically more favored course for complex intermediates as suggested by "substrate synergism" (21). Consistent with the latter role is the finding that there is conformational change accompanying phosphorylation of the enzyme by ATP (Chapter VII).

As we have studied in Chapters V and VI, the peptide mapping, subunit amino acid composition, qualitative and quantitative determination of the N-terminal of SCS confirm the $\alpha_2\beta_2$ subunit structure for this enzyme, with the smaller α subunit bearing the phosphohistidine. The observation of one phosphoryl group per mole of 140,000 daltons of SCS during phosphorylation may be explained as negative-cooperativity between two active phosphorylation sites or so-called "half-of-the-sites reactivity" (76). That is, phosphorylation of one subunit causes subsequent phosphorylation at the other apparently equivalent site to be less favorable. We have proposed a simple model based on the subunit structure and conformational change upon phosphorylation by ATP as depicted in Figure 17. Upon phosphorylation of either site of two α subunits, the enzyme will change to the compact form which cannot be further phosphorylated. This will stabilize the enzyme and protect it from proteolysis as detailed previously (Chapter VII). The phosphoryl group in the compact form may serve as a better leaving group in the catalysis. The dephosphorylation of the compact form will lead to the loose form which is easily attacked by proteases. Further phosphorylation of the compact form by ATP would be difficult, while phosphorylation of the loose form is proposed to lead to a conformational change to the compact form. There is an equilibrium between the compact form and the loose form of the

enzyme, and the amount of the compact form (phosphorylated form) may contribute to the stability of the enzyme. This is, of course, only a rough model to help us visualize a system consistent with the results known at the present time. The mechanism of this enzyme will be further investigated by means of transient kinetics and the enzyme-substrates complex studies as well as other techniques.

We have found that the rapid loss of activity of SCS following its dephosphorylation seems to be related to the presence of contaminating proteases. It is more likely some endo-proteases rather than exoproteases which break down the enzyme into smaller pieces easily. Indeed, the instability of the dephosphoenzyme in our hands has discouraged further in-depth studies of its structure-function relationships.



His represents the site of phosphorylation.

FIGURE 17: A simple model for conformational changes of SCS which accompany phosphorylation and dephosphorylation.

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APPENDIX

Publications arising from this work:

1. Wang, T., Jurasek, L., and Bridger, W. A., Biochemistry, 11, 2067 (1972).

2. Moffet, F. J., Wang, T., and Bridger, W. A., J. Biol. Chem., (in press).